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(54) THERMOTOLERANT RIBONUCLEASE H

(57) Polypeptides having an RNase H activity highly useful in genetic engineering; genes encoding these polypeptides; and a process for genetic engineeringly producing these polypeptides.

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Description

Technical Field

[0001] The present invention relates to a polypeptide, specifically a polypeptide having a ribonuclease H activity which is highly valuable for genetic engineering. The present invention also relates to a gene that is useful for producing said polypeptide by genetic engineering. The present invention further relates to a method for producing said polypeptide by genetic engineering.

Background Art

[0002] There are endo-type and exo-type ribonucleases (RNA-degrading enzymes). Their substrate specificities are various, and they are involved in complicated physiological activities. Enzymes such as ribonuclease T_1, ribonuclease T_2, ribonuclease H, ribonuclease P, ribonuclease I, ribonuclease II, ribonuclease III, ribonuclease IV, ribonuclease L are known to have ribonuclease activities.
[0003] Ribonuclease H (hereinafter also referred to as RNase H) was first isolated from calf thymus by W. H. Stein and P. Hausen in 1969. RNase Hs are currently classified into cellular RNase Hs and viral RNase Hs. The cellular RNase Hs are widely present in eukaryotes such as various animal cells and yeasts and prokaryotes such as Escherichia coli, whereas the viral RNase Hs are present in RNA tumor viruses. Several kinds of RNase H activities are present in a cell. They require divalent metal ions such as Mg^{2+} and Mn^{2+}.
[0004] An RNase H from Escherichia coli is a hydrolase that consists of 155 amino acids, has a molecular weight of about 17 kDa and has a substrate specificity of specifically cleaving only the RNA strand in a DNA-RNA hybrid in an endo-type manner. The resulting oligomer has a phosphate group at the 5' end and a hydroxyl group at the 3' end.
[0005] RNase HI and RNase HII have been identified as RNase Hs from E. coli. It has been shown that RNase HI has the following physiological functions in the replication of the Col E1 plasmid: 1) it degrades RNAs bound to portions other than the normal replication origin to ensure the normal replication origin; and 2) it synthesizes an RNA primer specific for the normal replication origin. The function of RNase HII remains unknown.
[0006] It is considered that RNase H increasingly becomes important with the development of genetic engineering. However, the expression level of this enzyme in E. coli is quite low. Then, production of this enzyme using recombinant DNA techniques has been attempted. RNase Hs produced using recombinant DNA techniques are now supplied from BRL, Amersham Pharmacia Biotech, Takara Shuzo and the like.
[0007] These commercially available recombinant RNase Hs are produced using Escherichia coli as a host (Kanaya et - al., The Journal of Biological Chemistry, 264:11546-11549 (1989)). A method of producing an RNase H from a thermophile, which is much more stable than RNase H from E. coli, using E. coli has been reported (Kanaya et al., Dai 2 Kai Nippon Tanpakukougakukai Nenkai Program/Abstract (1990) pp.69; Japanese Patent No. 2533671). However, the enzymatic activity of the RNase H from a thermophile produced using E. coli was lower than that of RNase H from E. coli.
[0008] RHase Hs have uses as exemplified below based on the substrate specificities, and attention is paid to RNase Hs as very valuable enzymes:

1) removal of template mRNA upon cDNA cloning;
2) removal of poly(A) region in mRNA; and
3) fragmentation of RNA.

[0009] However, as described above, only thermostable RNase Hs of which the productivities and the enzymatic activities are lower than those of RNase H from E. coli are available. Thus, development of a thermostable RNase H of which the productivity and the enzymatic activity are equivalent to or more than those of the RNase H from E. coli has been desired for expanding the uses of RNase H.

Objects of Invention

[0010] The main object of the present invention is to provide a polypeptide having an RNase H activity which is highly valuable for genetic engineering, a gene encoding said polypeptide and a method for producing said polypeptide by genetic engineering.

Summary of Invention

[0011] In view of the circumstances as described above, the present inventors have studied intensively and con-

ducted screening in order to obtain a thermostable RNase H. As a result, the present inventors have found a thermostable RNase H polypeptide having a high RNase H activity. Furthermore, the present inventors have found that the productivity of the thus obtained thermostable RNase H in production using genetic engineering techniques is high. Thus, the present invention has been completed.

[0012] The present invention is outlines as follows. The first aspect of the present invention relates to a thermostable ribonuclease H polypeptide, i.e., a polypeptide having a thermostable ribonuclease H activity, which is selected from the group consisting of:

(a) a polypeptide having the amino acid sequence of SEQ ID NO:9, 17, 23, 32, 37, 47, 57 or 59, or a portion thereof;
(b) a polypeptide having an amino acid sequence in which at least one amino acid residue is deleted, added, inserted or substituted in the amino acid sequence of SEQ ID NO:9, 17, 23, 32, 37, 47, 57 or 59; and
(c) a polypeptide having an amino acid sequence that shares at least 71% homology with the amino acid sequence of SEQ ID NO:9, 17, 23, 32, 37, 47, 57 or 59.

[0013] The second aspect of the present invention relates to a nucleic acid encoding a thermostable ribonuclease H, i.e., a nucleic acid encoding a polypeptide having a thermostable ribonuclease H activity, which is selected from the group consisting of:

(a) a nucleic acid encoding a polypeptide having the amino acid sequence of SEQ ID NO:9, 17, 23, 32, 37, 47, 57 or 59, or a portion thereof;
(b) a nucleic acid encoding a polypeptide having an amino acid sequence in which at least one amino acid residue is deleted, added, inserted or substituted in the amino acid sequence of SEQ ID NO:9, 17, 23, 32, 37, 47, 57 or 59;
(c) a nucleic acid having the nucleotide sequence of SEQ ID NO:8, 16, 22, 31, 36, 46, 56 or 58;
(d) a nucleic acid in which at least one nucleotide is deleted, added, inserted or substituted in the nucleotide sequence of SEQ ID NO:8, 16, 22, 31, 36, 46, 56 or 58 such that the deletion, addition, insertion or substitution of the nucleotide results in translation into an amino acid sequence;
(e) a nucleic acid that is hybridizable to any one of the nucleic acids of (a) to (d) or complementary strands thereof under stringent conditions; and
(f) a nucleic acid having a nucleotide sequence that shares at least 69% homology with the nucleotide sequence of SEQ ID NO:8, 16, 22, 31, 36, 46, 56 or 58.

[0014] The third aspect of the present invention relates to a recombinant DNA comprising the nucleic acid of the second aspect.

[0015] The fourth aspect of the present invention relates to a transformant transformed with the recombinant DNA of the third aspect.

[0016] The fifth aspect of the present invention relates to a method for producing a polypeptide having a thermostable ribonuclease H activity, the method comprising:

culturing the transformant of the fourth aspect; and
collecting a polypeptide having a thermostable ribonuclease H activity from the culture.

[0017] The sixth aspect of the present invention relates to a polypeptide having a thermostable ribonuclease H activity, which is obtainable by culturing a transformant into which any one of the plasmids pRHB11, pBCA3Nd2, pPFU220, pTM-RNH, pPHO238, pAFU204, pTLI204 and pTCE207 is transferred. Escherichia coli strains harboring these plasmids are deposited under Budapest Treaty at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology under accession numbers FERM BP-7655, FERM BP-7653, FERM BP-7654, FERM BP-7652, FERM BP-7692, FERM BP-7691, FERM BP-7693 and FERM BP-7694, respectively.

Detailed Description of the Invention

[0018] Hereinafter, the present invention will be described in detail.

[0019] As used herein, an RNase H refers to a hydrolase that has a substrate specificity of specifically cleaving only the RNA strand in a DNA-RNA hybrid in an endo-type manner, wherein the resulting oligomer has a phosphate group at the 5' end and a hydroxyl group at the 3' end.

[0020] Although it is not intended to limit the present invention, having a thermostable RNase H activity as used herein with respect to a polypeptide means that the polypeptide has an RNase H activity after incubating it at a temperature of 60°C or above for 15 minutes.

[0021] For example, a thermostable RNase H activity can be determined as follows.

[0022] 1 mg of poly(rA) or poly(dT) (both from Amersham Pharmacia Biotech) is dissolved in 1 ml of 40 mM tris-HCl (pH 7.7) containing 1 mM EDTA to prepare a poly(rA) solution and a poly(dT) solution.
[0023] The poly(rA) solution (to a final concentration of 20 ug/ml) and the poly(dT) solution (to a final concentration of 30 ug/ml) are then added to 40 mM tris-HCl (pH 7.7) containing 4 mM $MgCl_2$, 1 mM DTT, 0.003% BSA and 4% glycerol. The mixture is reacted at 37°C for 10 minutes and then cooled to 4°C at prepare a poly(rA)-poly(dT) solution.
[0024] 1 µl of an enzyme solution is added to 100 ul of the poly(rA)-poly(dT) solution. The mixture is reacted at 40°C for 10 minutes. 10 µl of 0.5 M EDTA is added thereto to terminate the reaction. Absorbance at 260 nm is then measured. As a control, 10 µl of 0.5 M EDTA is added to the reaction mixture, the resulting mixture is reacted at 40°C for 10 minutes, and the absorbance is then measured. A value (difference in absorbance) is obtained by subtracting the absorbance for the control from the absorbance for the reaction in the absence of EDTA. Thus, the concentration of nucleotide released from poly(rA)-poly(dT) hybrid by the enzymatic reaction is determined on the basis of the difference in absorbance. Thus, the thermostable RNase H activity according to the present invention can be determined.
[0025] Alternatively, the thermostable RNase H activity according to the present invention can be determined as follows. 100 µl of a reaction mixture [20 mM HEPES-potassium hydroxide (pH 8.5), 0.01% bovine serum albumin (Takara Shuzo), 1% dimethyl sulfoxide, 4 mM magnesium acetate, 20 µg/ml poly(dT) (Amersham Pharmacia Biotech), 30 ug/ml poly(rA) (Amersham Pharmacia Biotech)] which has been incubated at 40°C is added to 1 µl of an enzyme solution of which the activity is to be determined. The mixture is reacted at 40°C for 10 minutes. The reaction is then terminated by adding 10 µl of 0.5 M EDTA (pH 8.0). Absorbance at 260 nm is then measured.
[0026] One unit of an RNase H is defined as an amount of enzyme that increases A_{260} corresponding to release of 1 nmol of ribonucleotide in 10 minutes calculated according to the following equation:

Unit = [Difference in Absorbance x Reaction

Volume (ml)] / 0.0152

[0027] The polypeptides of the present invention include a polypeptide having an amino acid sequence in which at least one amino acid residue is deleted, added, inserted or substituted in the amino acid sequence of SEQ ID NO:9, 17, 23, 32, 37, 47, 57 or 59 as long as it exhibits a thermostable RNase H activity.
[0028] A mutation such as deletion, insertion, addition or substitution of an amino acid in an amino acid sequence may be generated in a naturally occurring polypeptide. Such mutation may be generated due to a polymorphism or a mutation of the DNA encoding the polypeptide, or due to a modification of the polypeptide in vivo or during purification after synthesis. However, it is known that such a mutated polypeptide may exhibit a physiological or biological activity substantially equivalent to that of a polypeptide without a mutation if such a mutation is present in a portion that is not important for the retention of the activity or the structure of the polypeptide.
[0029] This is applicable to a polypeptide in which such a mutation is artificially introduced into an amino acid sequence of a polypeptide. In this case, it is possible to generate more various mutations. For example, it is known that a polypeptide in which a cysteine residue in the amino acid sequence of human interleukin-2 (IL-2) is replaced by a serine retains the interleukin-2 activity (Science, 224:1431 (1984)).
[0030] Furthermore, it is known that certain polypeptides have peptide regions that are not indispensable to their activities. Such peptide regions are exemplified by a signal peptide in a polypeptide to be secreted extracellularly, or a prosequence or pre-prosequence found in a precursor of a protease. Most of such regions are removed after translation or upon conversion into an active polypeptide. Such a polypeptide has a primary structure different from that of a polypeptide without the region to be removed, but finally exhibits an equivalent function.
[0031] Genes having nucleotide sequences of SEQ ID NOS:8, 16, 22, 31, 36, 46, 56 or 58 which are isolated according to the present invention encode polypeptides having the amino acid sequences of SEQ ID NOS:9, 17, 23, 32, 37, 47, 57 or 59, respectively. These polypeptides have thermostable RNase H activities. The polypeptides of the present invention include polypeptides from which peptide regions that are not indispensable to their activities have been deleted therefrom.
[0032] When a polypeptide is produced by genetic engineering, a peptide chain that is irrelevant to the activity of the polypeptide of interest may be added at the amino terminus or the carboxyl terminus of the polypeptide. For example, a fusion polypeptide, in which a portion of an amino terminus region of a polypeptide that is expressed at a high level in the host to be used is added at the amino terminus of the polypeptide of interest, may be prepared in order to increase the expression level of the polypeptide of interest. In another case, a peptide having an affinity for a specific substance may be added at the amino terminus or the carboxyl terminus of the polypeptide of interest in order to facilitate the purification of the expressed polypeptide. The added peptide may remain added if it does not have a harmful influence on the activity of the polypeptide of interest. If necessary, it may be engineered such that it can be removed from the polypeptide of interest by appropriate treatment, for example, by limited digestion with a protease.

[0033] Thus, a polypeptide having an amino acid sequence in which at least one amino acid residue is deleted, inserted, added or substituted in the amino acid sequence of SEQ ID NO:9, 17, 23, 32, 37, 47, 57 or 59 disclosed herein is encompassed by the present invention if it has a thermostable RNase H activity.

[0034] Furthermore, a polypeptide having an amino acid sequence that shares at least 71%, preferably 80%, more preferably 90% homology with the amino acid sequence of SEQ ID NO:9, 17, 23, 32, 37, 47, 57 or 59 disclosed herein is encompassed by the present invention if it has a thermostable RNase H activity.

[0035] The homology can be determined using, for example, a computer program DNASIS-Mac (Takara Shuzo), a computer algorithm FASTA (version 3.0; Pearson, W. R. et al., Pro. Natl. Acad. Sci., 85:2444-2448, 1988) or a computer algorithm BLAST (version 2.0, Altschul et al., Nucleic Acids Res. 25:3389-3402, 1997).

[0036] For example, a polypeptide that shares at least 44% homology with the amino acid sequence of the ribonuclease HII from Bacillus caldotenax (SEQ ID NO:9), at least 47% homology with the amino acid sequence of the ribonuclease HIII from Bacillus caldotenax (SEQ ID NO:17), at least 69% homology with the amino acid sequence of the ribonuclease H from Pyrococcus furiosus (SEQ ID NO:23), at least 53% homology with the amino acid sequence of the ribonuclease H from Thermotoga maritima (SEQ ID NO:59) at least 51% homology with the amino acid sequence of the ribonuclease H from Archaeoglobus fulgidus (SEQ ID NO:37), at least 65% homology with the amino acid sequence of the ribonuclease H from Thermococcus litoralis (SEQ ID NO:47), at least 71% homology with the amino acid sequence of the ribonuclease H from Thermococcus celer (SEQ ID NO:57), or at least 71% homology with the amino acid sequence of the ribonuclease H from Pyrococcus horikoshii (SEQ ID NO:32) is encompassed by the present invention if it has a thermostable RNase H activity.

[0037] The polypeptide of the present invention can be produced, for example, by (1) purification from a culture of a microorganism producing the polypeptide of the present invention, or (2) purification from a culture of a transformant containing a nucleic acid encoding the polypeptide of the present invention.

(1) Purification from culture of microorganism producing the polypeptide of the present invention

[0038] The microorganism producing the polypeptide of the present invention is exemplified by Bacillus caldotenax (DSM406), Pyrococcus furiosus (DSM3638), Thermotoga maritima (DSM3109), Archaeoglobus fulgidus (DSM4139), Thermococcus litoralis (DSM5473) or Thermococcus celer (DSM2476) which can be purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, or Pyrococcus horikoshii (JCM9974) which can be purchased from the Institute of Physical and Chemical Research (RIKEN). The microorganism is cultured under conditions suitable for the growth of the microorganism. Preferably, culture conditions that increase the expression level of the polypeptide of interest are used. The polypeptide of interest produced in the cells or the culture medium can be purified according to a method conventionally used for purifying a protein.

[0039] A method conventionally used for culturing a thermostable bacterium can be utilized for the cultivation of the above-mentioned strain. Nutrients that can be utilized by the strain are added to the culture medium. For example, starch can be used as a carbon source, and Tryptone, peptone and yeast extract can be used as nitrogen sources. A metal salt such as a magnesium salt, a sodium salt or an iron salt may be added to a culture medium as a trace element. In addition, it may be advantageous to use artificial seawater for the preparation of a culture medium in case of a thermostable marine bacterium, for example.

[0040] The culture may be a standing culture or a spinner culture. For example, a dialysis culture method as described in Applied and Environmental Microbiology, 55:2086-2088 (1992) may be used. It is preferable to determine the culture conditions and the cultivation time depending on the strain or the composition of the culture medium to be used such that the productivity of the polypeptide becomes maximal.

[0041] A cell-free extract is first prepared in order to obtain a polypeptide. The cell-free extract can be prepared, for example, by collecting cells from a culture by centrifugation, filtration or the like and then disrupting the cells. A cell disruption method highly effective for extracting the enzyme of interest may be selected from sonication, disruption using beads, treatment with a lytic enzyme and the like. If the polypeptide is secreted into a culture supernatant, the polypeptide in the culture supernatant is concentrated by ammonium sulfate precipitation, ultrafiltration or the like. The concentrated polypeptide is used as a cell-free extract. A method conventionally used for purifying a protein can be used to isolate the polypeptide from the thus obtained cell-free extract. For example, ammonium sulfate precipitation, ion exchange chromatography, hydrophobic chromatography, gel filtration chromatography and the like can be used in combination.

(2) Purification from culture of transformant transformed with recombinant DNA containing nucleic acid encoding the polypeptide of the present invention

[0042] The polypeptide of the present invention can be obtained from a transformant transformed with a recombinant DNA that contains a nucleic acid encoding the polypeptide of the present invention, for example, a nucleic acid having

a nucleotide sequence of SEQ ID NO:8, 16, 22, 31, 36, 46, 56 or 58. A polypeptide having an amino acid sequence of SEQ ID NO:9 is produced using a nucleic acid having a nucleotide sequence of SEQ ID NO:8. A polypeptide having an amino acid sequence of SEQ ID NO:17 is produced using a nucleic acid having a nucleotide sequence of SEQ ID NO:16. A polypeptide having an amino acid sequence of SEQ ID NO:23 is produced using a nucleic acid having a nucleotide sequence of SEQ ID NO:22. A polypeptide having an amino acid sequence of SEQ ID NO:32 is produced using a nucleic acid having a nucleotide sequence of SEQ ID NO:31. A polypeptide having an amino acid sequence of SEQ ID NO:37 is produced using a nucleic acid having a nucleotide sequence of SEQ ID NO:36. A polypeptide having an amino acid sequence of SEQ ID NO:47 is produced using a nucleic acid having a nucleotide sequence of SEQ ID NO:46. A polypeptide having an amino acid sequence of SEQ ID NO:57 is produced using a nucleic acid having a nucleotide sequence of SEQ ID NO:56. A polypeptide having an amino acid sequence of SEQ ID NO:59 is produced using a nucleic acid having a nucleotide sequence of SEQ ID NO:58.

[0043] The polypeptide of the present invention may be purified from a culture obtained by culturing a transformant into which any one of the plasmids of the present invention pRHB11, pBCA3Nd2, pPFU220, pTM-RNH, pPHO238, pAFU204, pTLI204 and pTCE207 is transferred.

[0044] The host to be transformed is not limited to specific one and exemplified by those conventionally used in a field of recombinant DNA including Escherichia coli, Bacillus subtilis, yeast, filamentous fungi, plants, animals, cultured plant cells and cultured animal cells.

[0045] For example, the polypeptide of the present invention can be obtained using Escherichia coli harboring a plasmid in which the nucleic acid of the present invention is linked downstream from a lac promoter or a T7 phage promoter under conventional culture conditions, for example, in LB medium (10 g/l Tryptone, 5g/l yeast extract, 5 g/l NaCl, pH 7.2) containing 100 μg/ml of ampicillin at 37°C until logarithmic growth phase, adding isopropyl-β-D-thiogalactopyranoside at a final concentration of 1 mM thereto and further culturing at 37°C to express the polypeptide in the cultured cells.

[0046] Cells are collected by centrifugation after cultivation, disrupted by sonication, and a supernatant collected by centrifugation is used as a cell-free extract. This cell-free extract exhibits a thermostable RNase H activity. The polypeptide of the present invention can be purified from the cell-free extract by using known methods such as ion exchange chromatography, gel filtration, hydrophobic chromatography and ammonium sulfate precipitation. Naturally, a partially purified product obtained during the purification process as described above also exhibits an RNase H activity. Since the polypeptide of the present invention expressed in Escherichia coli harboring a plasmid linked to the nucleic acid of the present invention is thermostable, the cultured cells and/or the cell-free extract may be heated, for example, at a temperature of 40°C or above for about 10 minutes to remove heat-denatured insoluble proteins derived from the host in order to purify the polypeptide. An optimal temperature or time may be suitably selected for the heat treatment.

[0047] As described above, when the polypeptide of the present invention is expressed at normal temperature (e. g., 37°C) using a transformant harboring a nucleic acid encoding the polypeptide, the resulting expression product retains the activity, the thermostability and the like. That is, the polypeptide of the present invention can assume its inherent higher-order structure even if it is expressed at a temperature quite different from the growth temperature of the original producer cell.

[0048] The nucleic acid of the present invention is a nucleic acid that encodes the polypeptide of the present invention. Specifically, it is (1) a nucleic acid that encodes a polypeptide having the amino acid sequence of SEQ ID NO:9, 17, 23, 32, 37, 47, 57 or 59, or an amino acid sequence in which at least one amino acid residue is deleted, added, inserted or substituted in the sequence and having a thermostable RNase H activity; (2) a nucleic acid having the nucleotide sequence of SEQ ID NO:8, 16, 22, 31, 36, 46, 56 or 58; (3) a nucleic acid that has a nucleotide sequence that is hybridizable to the nucleotide sequence of (1) or (2) above under stringent conditions, or that shares at least 69%, preferably 80%, more preferably 90% homology with the nucleotide sequence of (1) or (2) above, and that encodes a polypeptide having a thermostable RNase H activity, or the like.

[0049] The homology of the nucleotide sequence can be determined using a computer program DNASIS-Mac, or a computer algorithm FASTA (version 3.0) or BLAST (version 2.0).

[0050] As used herein, a nucleic acid means a single-stranded or double-stranded DNA or RNA. If the nucleic acid of (2) above is an RNA, it is represented by a nucleotide sequence in which T is replaced by U in the nucleotide sequence of SEQ ID NO:8, for example.

[0051] For example, the nucleic acid of the present invention can be obtained as follows.

[0052] The nucleic acid of (2) above having the nucleotide sequence of SEQ ID NO:8, 16, 22, 31, 36, 46, 56 or 58 can be isolated as follows. A genomic DNA is prepared according to a conventional method from Bacillus caldotenax (DSM406), Pyrococcus furiosus (DSM3638), Thermotoga maritima (DSM3109), Archaeoglobus fulgidus (DSM4139), Thermococcus litoralis (DSM5473), Thermococcus celer (DSM2476) or Pyrococcus horikoshii (JCM9974) cultured as described above for the polypeptide of the present invention. The genomic DNA is used to construct a DNA library. The nucleic acid can be isolated from the DNA library. Also, the nucleic acid can be obtained by amplifying a nucleic acid having a nucleotide sequence of SEQ ID NO:8, 16, 22, 31, 36, 46, 56 or 58 by a polymerase chain reaction (PCR)

using the genomic DNA as a template.

[0053] Furthermore, a nucleic acid encoding a polypeptide having a thermostable RNase H activity similar to that of the polypeptide of the present invention can be obtained on the basis of the nucleotide sequence of the nucleic acid encoding the polypeptide of the present invention provided by the present invention (e.g., the nucleotide sequence of SEQ ID NO:8, 16, 22, 31, 36, 46, 56 or 58). Specifically, a DNA encoding a polypeptide having a thermostable RNase H activity can be screened by using the nucleic acid encoding the polypeptide of the present invention or a portion of the nucleotide sequence as a probe for hybridization from a DNA extracted from cells or PCR products obtained using the DNA as a template. Alternatively, a DNA encoding a polypeptide having a thermostable RNase H activity can be amplified using a gene amplification method such as a PCR using a primer designed based on the above-mentioned nucleotide sequence. Additionally, a DNA encoding a polypeptide having a thermostable RNase H activity can be chemically synthesized. The nucleic acids of (1) or (3) above can be obtained according to such a method.

[0054] A nucleic acid fragment containing only a portion of the nucleic acid of interest may be obtained according to the above-mentioned method. In this case, the entire nucleic acid of interest can be obtained as follows. The nucleotide sequence of the obtained nucleic acid fragment is determined to confirm that the fragment is a portion of the nucleic acid of interest. Hybridization is carried out using the nucleic acid fragment or a portion thereof as a probe. Alternatively, a PCR is carried out using a primer synthesized on the basis of the nucleotide sequence of the nucleic acid fragment.

[0055] "Hybridize under stringent conditions" refers to being capable of hybridizing under conditions as described in T. Maniatis et al. (eds.), Molecular Cloning: A Laboratory Manual 2nd ed., Cold Spring Harbor Laboratory (1989), for example, under the following conditions. A membrane onto which a nucleic acid is immobilized is bincubated with a probe in 6 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) containing 0.5% SDS, 0.1% bovine serum albumin (BSA), 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400 and 0.01% denatured salmon sperm nucleic acid at b50°C for 12 to 20 hours. After incubation, the membrane is washed in 2 x SSC containing 0.5% SDS at 37°C while changing the SSC concentration down to 0.1 x and the btemperature up to 50°C until the signal from the immobilized nucleic acid can be distinguished from background, and the probe is then detected. The activity of the protein encoded by the thus obtained novel nucleic acid is determined as described above, thereby confirming whether or not the nucleic acid is the nucleic acid of interest.

[0056] If an oligonucleotide probe is used, "stringent conditions" refer to, for example, incubation at a temperature of [Tm - 25°C] overnight in a solution bcontaining 6 x SSC, 0.5% SDS, 5 x Denhardt's and 0.01% denatured salmon sperm nucleic acid although it is not intended to limit the present invention.

[0057] Tm of an oligonucleotide probe or primer can be determined, for example, according to the following equation:

$$Tm = 81.5 - 16.6(\log_{10}[Na^+]) + 0.41(\%G{+}C) - (600/N)$$

wherein N is the chain length of the oligonucleotide probe or primer; %G+C is the content of guanine and cytosine residues in the oligonucleotide probe or primer.

[0058] If the chain length of the oligonucleotide probe or primer is shorter than 18 bases, Tm can be estimated, for example, as the sum of the product of the number of A+T (adenine and thymine) residues multiplied by 2(°C) and the product of the number of G+C residues multiplied by 4(°C):

$$[(A+T) \times 2 + (G+C) \times 4].$$

[0059] According to the present invention, a nucleic acid which is capable of hybridizing to the nucleic acid encoding the polypeptide of the present invention under stringent conditions is encompassed by the present invention as long as it encodes a polypeptide having a thermostable RNase H activity even if it does not have the same nucleotide sequence as that disclosed herein, as described above.

[0060] It is known that one to six codon(s) (a combination of three bases), which defines an amino acid in a gene, is assigned for each amino acid. Thus, many nucleic acids can encode one specific amino acid sequence although it depends on the amino acid sequence. Nucleic acids are not necessarily stable in the nature. Generation of a mutation in a nucleotide sequence is not unusual. A mutation generated in a nucleic acid may not alter the encoded amino acid sequence (called a silent mutation). In this case, it can be said that a different nucleic acid encoding the same amino acid sequence is generated. Thus, it cannot be denied that various nucleic acids encoding the same amino acid sequence can be generated in the course of passage of an organism containing a nucleic acid encoding one specific amino acid sequence. Furthermore, it is not difficult to artificially produce various nucleic acids encoding the same amino acid sequence if one uses various genetic engineering techniques.

[0061] For example, if a codon used in an original nucleic acid encoding a protein of interest is one whose codon usage is low in the host to be used for producing the protein by genetic engineering, the expression level of the protein

may be low. In this case, the codon is artificially converted into one frequently used in the host without altering the encoded amino acid sequence aiming at elevating the expression level of the protein of interest (e.g., JP-B 7-102146). As described above, various nucleic acids encoding one specific amino acid sequence can be artificially prepared, of course. They may also be generated in the nature.

[0062] The nucleic acid encoding the polypeptide of the present invention (e.g., a nucleic acid having the nucleotide sequence of SEQ ID NO:7) can be ligated to an appropriate vector to construct a recombinant DNA. The vector to be used for the construction of the recombinant DNA is not specifically limited. For example, plasmid vectors, phage vectors and virus vectors can be used. A suitable vector for the object of the recombinant DNA is selected.

[0063] Furthermore, a transformant can be produced by introducing the recombinant DNA into an appropriate host. The host to be used for the production of a transformant is not specifically limited. Microorganisms such as bacteria, yeasts and filamentous fungi as well as cultured cells from animals, plants, insects and the like can be used. The polypeptide of the present invention can be produced in large quantities by culturing the transformant to produce the polypeptide of the present invention in the culture.

Examples

[0064] The following Examples illustrate the present invention in more detail, but are not to be construed to limit the scope thereof.

Example 1: Preparation of RNase H from thermophile Bacillus caldotenax

[0065] Bacillus caldotenax YT-G (purchased from Deutsche Sammlung von Mikroorganismen; DSM406) was inoculated into 100 ml of a medium containing 0.2% Tryptone (Difco Laboratories) and 1.5% yeast extract (Difco Laboratories) (pH 6.5), cultured at 60°C for 140 minutes with shaking and used as a pre-culture. 30 ml of the pre-culture was inoculated into 3 L of a medium having the same composition and cultured with aeration at 2.5 L/minute and stirring at 250 rpm at a temperature of 60°C for 5 hours.

[0066] The cells were collected by centrifuging the culture at 5000 x g for 15 minutes. 402 g (wet weight) of the cells were suspended in 1000 ml of 50 mM tris-HCl buffer (pH 7.5) containing 10 mM mercaptoethanol, 0.5 M NaCl, 1 mM EDTA and 20 uM PAPMSF and disrupted using MINI-Lab (APV GAULIN/RANNIE). Cell debris were removed by centrifugation to recover a supernatant.

[0067] A polyethylene imine solution was added to the resulting supernatant at a final concentration of 0.1%. After stirring, the mixture was allowed to stand for 1 hour. A supernatant was then recovered by centrifugation. Ammonium sulfate was added to the supernatant to 50% saturation. A precipitate obtained by centrifugation was dissolved in 20 mM tris-HCl buffer (pH 7.5) containing 10 mM mercaptoethanol, 0.1 mM EDTA, 50 mM NaCl and 10% glycerol. The solution was dialyzed against the same buffer. The dialyzed sample was loaded onto a 280-ml DE52 column (Whatman) equilibrated with the same buffer and non-adsorptive fractions were collected.

[0068] The column was further washed with 420 ml of the buffer used for the equilibration and washing fractions were collected. The non-adsorptive fractions and the washing fractions from the DE52 column chromatography were mixed together and loaded onto a 240-ml P-11 column (Whatman) equilibrated with 20 mM tris-HCl buffer (pH 7.5) containing 10 mM mercaptoethanol, 0.1 mM EDTA, 50 mM NaCl and 10% glycerol. Elution was then carried out using the equilibration buffer containing 0 to 0.5 M NaCl.

[0069] The resulting active fractions were placed in a dialysis tube. The tube was placed on solid polyethylene glycol 20000 for dehydration-concentration at 4°C. The enzyme concentrate was then loaded onto a 300-ml Superdex G-200 column (Amersham Pharmacia Biotech) equilibrated with 25 mM tris-HCl buffer (pH 7.5) containing 5 mM mercaptoethanol, 0.5 mM EDTA, 30 mM NaCl and 10% glycerol. Elution was carried out using the buffer used for equilibration to obtain active fractions. The active fractions were loaded onto a 15-ml Heparin-Sepharose column (Amersham Pharmacia Biotech) equilibrated with 20 mM tris-HCl buffer (pH 7.5) containing 10 mM mercaptoethanol, 0.1 mM EDTA, 50 mM NaCl and 10% glycerol. Elution was carried out using the equilibration buffer containing 0 to 0.5 M NaCl.

[0070] The resulting active fractions were loaded onto a 5-ml Hitrap-SP column (Amersham Pharmacia Biotech) equilibrated with 20 mM tris-HCl buffer (pH 7.5) containing 10 mM mercaptoethanol, 0.1 mM EDTA, 50 mM NaCl and 10% glycerol. Elution was carried out using the equilibration buffer containing 0 to 0.5 M NaCl The resulting active fractions were loaded onto a 300-ml Superdex G-200 column (Amersham Pharmacia Biotech) equilibrated with 25 mM tris-HCl buffer (pH 7.5) containing 5 mM mercaptoethanol, 0.5 mM EDTA, 30 mM NaCl and 10% glycerol again. The resulting active fractions were used as an RNase H preparation (an enzyme solution).

[0071] A thermostable RNase H activity was measured as follows.

[0072] 1 mg of poly(rA) or poly(dT) (both from Amersham Pharmacia Biotech) was dissolved in 1 ml of 40 mM tris-HCl (pH 7.7) containing 1 mM EDTA to prepare a poly(rA) solution and a poly(dT) solution.

[0073] The poly(rA) solution (to a final concentration of 20 μg/ml) and the poly(dT) solution (to a final concentration

of 30 μg/ml) were then added to 40 mM tris-HCl (pH 7.7) containing 4 mM $MgCl_2$, 1 mM DTT, 0.003% BSA and 4% glycerol. The mixture was reacted at 37°C for 10 minutes and then cooled to 4°C at prepare a poly(rA)-poly(dT) solution.

[0074] 1 μl of an enzyme solution was added to 100 μl of the poly(rA)-poly(dT) solution. The mixture was reacted at 40°C for 10 minutes. 10 μl of 0.5 M EDTA was added thereto to terminate the reaction. Absorbance at 260 nm was then measured. As a control, 10 μl of 0.5 M EDTA was added to the reaction mixture, the resulting mixture was reacted at 40°C for 10 minutes, and the absorbance was then measured. A value (difference in absorbance) was obtained by subtracting the absorbance for the control from the absorbance for the reaction in the absence of EDTA. Thus, the concentration of nucleotide released from poly(rA)-poly(dT) hybrid by the enzymatic reaction was determined on the basis of the difference in absorbance. One unit of an RNase H was defined as an amount of enzyme that increases A_{260} corresponding to release of 1 nmol of ribonucleotide in 10 minutes calculated according to the following equation. If a diluted enzyme solution is used, the value obtained using the following equation was corrected based on the dilution rate:

Unit = [Difference in Absorbance x Reaction

Volume (ml)] / 0.0152

Example 2: Cloning of Bacillus caldotenax RNase HII gene

(1) Preparation of genomic DNA from Bacillus caldotenax

[0075] Bacillus caldotenax YT-G (DSM406) was inoculated into 60 ml of LB medium (1% Tryptone, 0.5% yeast extract and 0.5% NaCl, pH 7.2) and cultured at 65 ° C for 20 hours. After culturing, the culture was centrifuged to collect-cells. The cells were suspended in 2 ml of 25% sucrose and 50 mM tris-HCl (pH 8.0). 0.2 ml of 10 mg/ml lysozyme chloride (Nacalai Tesque) in water was added thereto. The mixture was reacted at 20°C for 1 hour. After reaction, 12 ml of a mixture containing 150 mM Nacl, 1 mM EDTA and 20 mM tris-HCl (pH 8.0), 0.1 ml of 20 mg/ml proteinase K (Takara Shuzo) and 1 ml of a 10% aqueous solution of sodium lauryl sulfate were added to the reaction mixture. The mixture was incubated at 37°C for 1 hour.

[0076] 2.1 ml of 5 M NaCl and 2 ml of a CTAB-NaCl solution [10% cetyltrimethylammonium bromide (Nacalai Tesque) and 0.7 M NaCl] were then added to the mixture and the resulting mixture was mixed thoroughly and incubated at 65°C for 10 minutes. An equal volume of a mixture of chloroform/isoamyl alcohol (24 : 1, v/v) was added thereto. The resulting mixture was gently mixed for 10 minutes and then centrifuged for 10 minutes at 10,000 x g. After centrifugation, an equal volume of a mixture of phenol saturated with 100 mM tris-HCl (pH 8.0)/chloroform/isoamyl alcohol (25 : 24: 1, v/v) was added to the resulting supernatant. The resulting mixture was gently mixed for 10 minutes and then centrifuged for 10 minutes at 10,000 x g. After centrifugation, a 0.6 volume of 2-propanol was added to the resulting supernatant. The resulting fibrous precipitate was wound using a glass bar, washed with 70% ethanol in water, air-dried and then dissolved in 0.5 ml of TE buffer to obtain a genomic DNA solution.

(2) Cloning of middle portion of RNase HII gene

[0077] Oligonucleotides BsuII-3 and BsuII-6 represented by SEQ ID NOS:1 and 2 were synthesized on the basis of Motif I and Motif III, portions conserved among amino acid sequences of RNase HIIs from various organisms (Biochemistry, 38:605-608 (1999)).

[0078] A PCR was carried out in a volume of 100 μl using 1 μl of the Bacillus caldotenax genomic DNA solution as prepared in Example 2-(1) as a template, and 100 pmol each of BsuII-3 and BsuII-6 as primers. TaKaRa Taq (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 50 cycles of 94°C for 30 seconds, 45°C for 30 seconds and 72°C for 1 minute. After reaction, the reaction mixture was subjected to phenol treatment followed by ethanol precipitation to purify a DNA. The resulting DNA was blunt-ended using T4 DNA polymerase (Takara Shuzo) and then subjected to agarose gel electrophoresis to recover an amplified DNA fragment of about 0.4 kb from the gel. The about 0.4-kb DNA fragment was ligated with pUC119 (Takara Shuzo) digested with SmaI (Takara Shuzo) using T4 DNA ligase (Takara Shuzo). The ligation mixture was used to transform Escherichia coli JM109.

[0079] The resulting transformants were cultured to obtain a plasmid 21-12 into which the about 0.4-kb DNA fragment was inserted.

(3) Cloning of upstream portion of RNase HII gene

[0080] The nucleotide sequence of the inserted fragment of about 0.4 kb in the plasmid 21-12 obtained in Example 2-(2) was determined. Oligonucleotides RNII-S1 and RNII-S2 represented by SEQ ID NOS:3 and 4 were synthesized on the basis of the determined nucleotide sequence.

[0081] The Bacillus caldotenax genomic DNA as prepared in Example 2-(1) was digested with BamHI (Takara Shuzo) and ligated with a Sau3AI cassette (Takara Shuzo) using T4 DNA ligase. A procedure was carried out according to the protocol attached to TaKaRa LA PCR in vitro cloning kit (Takara Shuzo) using the ligation mixture as a template, RNII-S2 as a primer for a primary PCR and RNII-S1 as a primer for a secondary PCR. A DNA was purified from the solution after the secondary PCR by phenol extraction followed by ethanol precipitation. The DNA was blunt-ended using T4 DNA polymerase and then subjected to agarose gel electrophoresis to recover an amplified DNA fragment of about 1.5 kb from the gel. The about 1.5-kb DNA fragment was ligated with pUC119 digested with SmaI using T4 DNA ligase. The ligation mixture was used to transform Escherichia coli JM109.

[0082] The resulting transformants were cultured to obtain a plasmid B25N16 into which the about 1.5-kb DNA fragment was inserted.

(4) Cloning of entire RNase HII gene

[0083] Oligonucleotides RNII-S5 and RNII-S6 represented by SEQ ID NOS:5 and 6 were synthesized on the basis of the nucleotide sequence of the inserted fragment of about 0.4 kb in the plasmid 21-12 as determined in Example 2-(3).

[0084] A PCR was carried out using the plasmid 21-12 as prepared in Example 2-(2) as a template, and RNII-S5 and RNII-S6 as primers. TaKaRa Ex Taq (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. After reaction, the reaction mixture was subjected to agarose gel electrophoresis. An amplified DNA fragment of about 0.3 kb was recovered from the gel. The about 0.3-kb DNA fragment was labeled with digoxigenin using DIG High-prime (Roche Diagnostics).

[0085] Southern hybridization was carried out for digests of the Bacillus caldotenax genomic DNA as prepared in Example 2-(1) with HindIII (Takara Shuzo), Sac I (Takara Shuzo), or HindIII and SacI using the digoxigenin-labeled DNA as a probe.

[0086] Hybridization and detection were carried out using DIG Luminescent Detection Kit (Roche Diagnostics) according to the protocol attached thereto.

[0087] As a result, DNA fragments of about 4.5 kb, about 5.8 kb and about 1.3 kb were hybridized with the probe for the digests with HindIII, SacI, and HindIII and SacI, respectively.

[0088] Based on these results, the Bacillus caldotenax genomic DNA was digested with HindIII and subjected to agarose gel electrophoresis to recover DNA fragments of about 4.5 kb from the gel. The resulting DNA fragments were digested with SacI and subjected to agarose gel electrophoresis to recover DNA fragments of about 1.3 kb from the gel. The resulting DNA fragments were ligated with pUC19 (Takara Shuzo) digested with HindIII and SacI using T4 DNA ligase. The ligation mixture was used to transform Escherichia coli HB101.

[0089] The resulting transformants were replica-plated onto Hybond-N (Amersham Pharmacia Biotech). Colony hybridization was then carried out using the above-mentioned digoxigenin-labeled probe according to a conventional method. A plasmid pRHB1 was prepared from the thus obtained positive clone.

[0090] The nucleotide sequence of the DNA inserted into pRHB1 was then determined. Comparison of the amino acid sequence deduced from the nucleotide sequence with the amino acid sequence of the RNase HIT from Bacillus subtilis suggested that a region of about 40 bp from the initiation codon was missing in the DNA in pRHB1. Then, the full length RNase HII gene was constructed as follows.

[0091] B25N16 as prepared in Example 2-(3) was digested with HindIII and subjected to agarose gel electrophoresis to recover a DNA fragment of about 160 bp from the gel. The about 160-bp DNA fragment was ligated with pRHB1 digested with HindIII using T4 DNA ligase. The ligation mixture was used to transform Escherichia coli HB101. Plasmids were prepared from the resulting transformants.

[0092] Next, an oligonucleotide RNII-Nde represented by SEQ ID NO:7 was synthesized on the basis of the presumed nucleotide sequence around the initiation codon. PCRs were carried out using the plasmids prepared from the transformants as templates, and RNII-Nde and RNII-S6 as primers. A plasmid from which a DNA fragment of about 0.7 kb was amplified was selected and designated as pRHB11.

[0093] The nucleotide sequence of the DNA fragment inserted into the thus obtained plasmid pRHB11 was determined. Analysis of the results revealed an open reading frame (ORF) presumably encoding RNase HII. The nucleotide sequence of this open reading frame is shown in SEQ ID NO:8. The amino acid sequence of RNase HII deduced from the nucleotide sequence is shown in SEQ ID NO:9.

[0094] Escherichia coli HB101 transformed with the plasmid pRHB11 is designated and indicated as Escherichia coli

HB101/pRHB11, and deposited on September 5, 2000 (date of original deposit) at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566, Japan under accession number FERM BP-7655.

(5) Expression of Bacillus caldotenax RNase HII gene

[0095] Escherichia coli HB101 transformed with pRHB11 or pRHB1 was inoculated into 5 ml of LB medium containing 100 μg/ml of ampicillin and cultured with shaking at 37°C overnight. After cultivation, cells collected by centrifugation were suspended in 0.5 ml of TE buffer and sonicated. A supernatant obtained by centrifugation was used as a crude cell extract.
[0096] 10 mM tris-HCl (pH 8.0), 1 mM DTT (Nacalai Tesque), 0.003% BSA (fraction V, Sigma), 4% glycerol, 20 μg/ml poly(dT) (Amersham Pharmacia Biotech) and 30 μg/ml poly(rA) (Amersham Pharmacia Biotech) were mixed together. The mixture was incubated at 37°C for 10 minutes and used as a substrate solution for measuring an RNase H activity.
[0097] 1 μl of 1 M $MnCl_2$ was added to 100 μl of the substrate solution. The mixture was incubated at 40°C. 10 μl of a 10-fold dilution of the crude cell extract was added to the mixture to initiate a reaction. After reacting at 40°C for 30 minutes, 10 μl of 0.5 M EDTA was added thereto to terminate the reaction. Absorbance at 260 nm was then measured.
[0098] As a result, the absorbance at 260 nm from a reaction in which a crude cell extract prepared from Escherichia coli HB101 harboring pRHB11 was used was clearly higher than that from a reaction in which a crude cell extract prepared from Escherichia coli HB101 harboring pRHB1 was used. Thus, it was demonstrated that pRHB1 contained an RNase HII gene and that Escherichia coli harboring pRHB11 expressed an RNase H activity.

(6) Preparation of purified RNase HII preparation

[0099] Escherichia coli HB101 transformed with pRHB11 obtained in Example 2-(4) was inoculated into 1 L of LB medium containing 100 μg/ml of ampicillin and cultured with shaking at 37°C for 16 hours. After cultivation, cells collected by centrifugation were suspended in 52.3 ml of a sonication buffer [50 mM tris-HCl (pH 8.0), 2 mM 2-mercaptoethanol, 10% glycerol, 2 mM phenylmethanesulfonyl fluoride] and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12,000 rpm for 10 minutes was heated at 60°C for 15 minutes. It was then centrifuged again at 12,000 rpm for 10 minutes to collect a supernatant. Thus, 50.0 ml of a heated supernatant was obtained.
[0100] The solution was subjected to RESOURSE Q column (Amersham Pharmacia Biotech) equilibrated with Buffer C [50 mM tris-HCl (pH 8.0), 2 mM 2-mercaptoethanol, 10% glycerol] and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HII flowed through the RESOURSE Q column.
[0101] 51 ml of the flow-through RNase HII fraction was subjected to RESOURSE S column (Amersham Pharmacia Biotech) equilibrated with Buffer C and eluted with a linear gradient of 0 to 500 mM NaCl using FPLC system. A fraction containing RNase HII eluted with about 240 mM NaCl was obtained.
[0102] 3.0 ml of the RNase HII fraction was subjected in two portions to PD-10 column (Amersham Pharmacia Biotech) equilibrated with Buffer C containing 50 mM NaCl. 7.0 ml the resulting eluate was subjected to HiTrap-heparin column (Amersham Pharmacia Biotech) equilibrated with Buffer C containing 50 mM NaCl and eluted with a linear gradient of 50 to 550 mM NaCl using FPLC system. A fraction containing RNase HII eluted with about 310 mM NaCl was obtained.
[0103] 4.4 ml of the RNase HII fraction was concentrated by ultrafiltration using Centricon-10 (Amicon). 280 μl of the concentrate was subjected to Superdex 200 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 50 mM tris-HCl (pH 8.0) containing 100 mM NaCl and 0.1 mM EDTA and eluted with the same buffer. As a result, RNase HII was eluted at a position corresponding to a molecular weight of 35 kilodalton. This molecular weight corresponds to that of the RNase HII in the monomeric form.
[0104] The thus eluted RNase HII was used as Bca RNase HII preparation.
[0105] The enzymatic activity of the thus obtained Bca RNase HII preparation was measured as follows.
[0106] 100 μl of a reaction mixture [20 mM HEPES-potassium hydroxide (pH 7.8), 0.01% bovine serum albumin (Takara Shuzo), 1% dimethyl sulfoxide, 10 mM manganese chloride, 20 μg/ml poly(dT) (Amersham Pharmacia Biotech), 30 μg/ml poly(rA) (Amersham Pharmacia Biotech)] which had been incubated at 40°C was added to 1 μl of the Bca RNase HII preparation. The mixture was reacted at 40°C for 10 minutes. The reaction was then terminated by adding 10 μl of 0.5 M EDTA (pH 8.0). Absorbance at 260 nm was then measured.
[0107] As a result, an RNase H activity was observed for the Bca RNase HII preparation.

Example 3: Cloning of Bacillus caldotenax RNase HIII gene

(1) Cloning of fragment of RNase HIII gene

[0108] Primers BsuIII-1, BsuIII-3, BsuIII-6 and BsuIII-8 represented by SEQ ID NOS:10 to 13 for screening a gene encoding RNase HIII were synthesized based on the amino acid sequences of regions well conserved among Bacillus subtilis and other organisms determined on the basis of the homology among the amino acid sequences of RNase HIIIs from Bacillus subtilis [Otani, N. et al., Biochemistry, 38:605-608 (1999)] and other organisms.

[0109] A first PCR was carried out in a volume of 50 µl using 200 ng of the Bacillus caldotenax genomic DNA as prepared in Example 2-(1) as a template, and 100 pmol each of BsuIII-1 and BsuIII-8 as primers. A second PCR was then carried out in a volume of 100 µl using 1 µl of the reaction mixture as a template, and 100 pmol each of BsuIII-3 and BsuIII-6 as primers. TaKaRa Taq polymerase (Takara Shuzo) was used as a DNA polymerase for the two PCRs according to the attached protocol. The PCRs were carried out as follows: 25 (the first PCR) or 30 (the second PCR) cycles of 94°C for 30 seconds, 45°C for 30 seconds and 72°C for 1 minute.

[0110] An amplified DNA fragment of about 450 bp was blunt-ended using T4 DNA polymerase (Takara Shuzo) and then subjected to agarose gel electrophoresis to recover the amplified DNA fragment of about 450 bp. The about 450-bp DNA fragment was ligated with pUC119 (Takara Shuzo) digested with SmaI (Takara Shuzo) using T4 DNA ligase (Takara Shuzo). The ligation mixture was used to transform Escherichia coli JM109.

[0111] The resulting transformants were cultured to obtain a plasmid pBCA3204 into which the about 450-bp DNA fragment was inserted.

(2) Cloning of RNase HIII gene using Southern hybridization method

[0112] The nucleotide sequence of the DNA fragment inserted in pBCA3204 obtained in Example 3-(1) was determined. Primers RNIII-S3 and BcaRNIII-3 represented by SEQ ID NOS:14 and 15 were synthesized on the basis of the determined nucleotide sequence. A PCR was carried out in a volume of 100 µl using RNIII-S3 and BcaRNIII-3 as primers and pBCA3204 as a template. TaKaRa Z-Taq (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 30 cycles of 98°C for 0 second, 55°C for 0 second and 72°C for 20 seconds. After reaction, the reaction mixture was subjected to phenol-chloroform extraction, ethanol precipitation and agarose gel electrophoresis to recover a DNA fragment of about 0.4 kb from the gel. The about 0.4-kb DNA fragment was labeled using DIG DNA Labeling Kit (Boehringer Mannheim) to prepare a probe.

[0113] 20 µg of the Bacillus caldotenax genomic DNA prepared in Example 2-(1) was completely digested with BamHI, EcoRI, HindIII, PstI or XbaI (all from Takara Shuzo). The half of each of the digests was then subjected to agarose gel electrophoresis. The DNAs were transferred from the agarose gel to a nylon membrane using 0.4 N NaOH and fixed at 120°C for 30 minutes. The membrane was preincubated in a sealed bag containing 30 ml of a hybridization buffer [43.4 g/L sodium chloride, 17.6 g/L sodium citrate, 1% blocking agent (Boehringer Mannheim), 0.1% N-lauroyl sarcosine, 0.02% sodium lauryl sulfate (SDS)] at 60°C for 4 hours and then incubated in a sealed bag containing 5 ml of a hybridization buffer containing the probe at 60°C for 16 hours.

[0114] The membrane was washed twice in 50 ml of 2 x SSC (17.5 g/L NaCl, 7.7 g/L sodium citrate) containing 0.1% SDS at room temperature, and twice in 50 ml of 0.5 x SSC (4.4 g/L sodium chloride, 1.9 g/L sodium citrate) containing 0.1% SDS at 45°C. Then, an EcoRI fragment of about 8 kb, a PstI fragment of about 4.5 kb and a HindIII fragment of about 1 kb which have sequences complementary to the probe were detected using DIG nucleic acid detection kit (Boehringer Mannheim).

[0115] The remaining half of the Bacillus caldotenax genomic DNA completely digested with PstI was subjected to agarose gel electrophoresis. PstI fragments of about 4.5 kb were recovered from the gel. The DNA fragments were then ligated with a plasmid vector pTV119N, which had been digested with PstI and dephosphorylated with alkaline phosphatase (Takara Shuzo). The ligation mixture was used to transform Escherichia coli JM109.

[0116] A PCR was carried out in a volume of 50 µl using RNIII-S3 and BcaRNIII-3 as primers, and one of the colonies as a template to select a colony presumably harboring an RNase HIII gene. TaKaRa-Z Taq (Takara Shuzo) was used for the PCR according to the attached protocol. The PCR was carried out as follows: 30 cycles of 98°C for 0 second, 55°C for 0 second and 72°C for 20 seconds. As a result, it was found that the gene of interest was contained in the colony No. 88.

[0117] A PCR was carried out using a plasmid prepared from the colony No. 88 as a template, and a primer pair RN-N (Takara Shuzo) and BcaRNIII-3 or a primer pair M4 (Takara Shuzo) and RNIII-S3 to examine whether or not the entire RNase HIII gene was contained in the plasmid. As a result, it was anticipated based on the length of the amplification product that the entire RNase HIII gene was contained in the plasmid, which was designated as pBCA3P88.

(3) Determination of nucleotide sequence of DNA fragment containing RNase HIII gene

[0118] The nucleotide sequence of the DNA fragment inserted into the plasmid pBCA3P88 obtained in Example 3-(2) was determined according to a dideoxy method.

[0119] Analysis of the determined nucleotide sequence revealed the existence of an open reading frame encoding an amino acid sequence including the N-terminal amino acid sequence of RNase HIII. The nucleotide sequence of the open reading frame and the amino acid sequence of RNase HIII deduced from the nucleotide sequence are shown in SEQ ID NO:16 and SEQ ID NO:17, respectively.

(4) Construction of plasmid for expressing RNase HIII

[0120] A PCR was carried out in a volume of 100 μl using the plasmid pBCA3P88 as described in Example 3-(2) as a template, BcaRNIIINde represented by SEQ ID NO:18 designed with reference to the sequence around the above-mentioned open reading frame for RNase HIII and M13 primer M4 (Takara Shuzo). Pyrobest DNA polymerase (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 3 minutes. An amplified DNA fragment of about 4 kb was digested with NdeI (Takara Shuzo) and subjected to agarose gel electrophoresis to recover an NdeI fragment of about 1.4 kb from the gel. The about 1.4-kb DNA fragment was ligated with pTV119Nd (a plasmid in which the NcoI site in pTV119N is converted into a NdeI site) which had been digested with NdeI and dephosphorylated with alkaline phosphatase (Takara Shuzo). The ligation mixture was used to transform Escherichia coli JM109.

[0121] Next, a PCR was carried out in a volume of 50 μl using one of the colonies as a template, and RV-N (Takara Shuzo) and BcaRNIII-3 (SEQ ID NO:15) as primers in order to screen for a plasmid in which the RNase HIII gene in the NdeI fragment was linked downstream from the lac promoter in the vector pTV119Nd. A colony presumably harboring the RNase HIII gene was then selected. TaKaRa-Z Taq (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 30 cycles of 98°C for 0 second, 55°C for 0 second and 72°C for 20 seconds. As a result, it was found that the colony No. 2 contained a plasmid in which the RNase HIII gene in the NdeI fragment was linked downstream from the lac promoter in the vector pTV119Nd. This plasmid was designated as pBCA3Nd2.

[0122] The determination of the nucleotide sequence of the DNA fragment inserted into the plasmid by a dideoxy method revealed that there was no mutation due to the PCR except that the initiation codon GTG was changed to ATG.

[0123] Escherichia coli JM109 transformed with the plasmid pBCA3Nd2 is designated and indicated as Escherichia coli JM109/pBCA3Nd2, and deposited on September 5, 2000 (date of original deposit) at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305-8566, Japan under accession number FERM BP-7653.

(5) Preparation of purified RNase HIII preparation

[0124] Escherichia coli JM109 transformed with pBCA3Nd2 obtained in Example 3-(4) was inoculated into 2 L of LB medium containing 100 μg/ml of ampicillin and cultured with shaking at 37°C for 16 hours. After cultivation, cells collected by centrifugation were suspended in 39.6 ml of a sonication buffer [50 mM tris-HCl (pH 8.0), 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride] and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12,000 rpm for 10 minutes was heated at 60°C for 15 minutes. It was then centrifuged again at 12,000 rpm for 10 minutes to collect a supernatant. Thus, 39.8 ml of a heated supernatant was obtained.

[0125] The heated supernatant was subjected to RESOURSE Q column (Amersham Pharmacia Biotech) equilibrated with Buffer A [50 mM tris-HCl (pH 8.0), 1 mM EDTA] and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HIII flowed through the RESOURSE Q column.

[0126] 45 ml of the flow-through RNase HIII fraction was dialyzed against 2 L of Buffer B [50 mM tris-HCl (pH 7.0), 1 mM EDTA] for 2 hours. The dialysis was repeated for two more times under the same conditions. 55.8 ml of the dialyzed enzyme solution was subjected to RESOURSE S column (Amersham Pharmacia Biotech) equilibrated with Buffer B and eluted with a linear gradient of 0 to 500 mM NaCl using FPLC system. A fraction containing RNase HIII eluted with about 105 mM NaCl was obtained.

[0127] Buffer B containing 1 M NaCl was added to 7.0 ml of the fraction to make the NaCl concentration to 150 mM. The mixture was subjected to HiTrap-heparin column (Amersham Pharmacia Biotech) equilibrated with Buffer B containing 150 mM NaCl. As a result, RNase HIII flowed through the HiTrap-heparin column.

[0128] 7.5 ml of the flow-through RNase HIII fraction was concentrated by ultrafiltration using Centricon-10 (Millipore). 190 μl of the concentrate was subjected to Superdex 200 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 50 mM tris-HCl (pH 7.0) containing 100 mM NaCl and 0.1 mM EDTA and eluted with the same buffer. As a result, RNase HIII was eluted at a position corresponding to a molecular weight of 33 kilodalton. This molecular

weight corresponds to that of the RNase HIII in the monomeric form.

[0129] The thus eluted RNase HIII was used as Bca RNase HIII preparation.

[0130] The enzymatic activity of the thus obtained Bca RNase HIII preparation was measured as follows.

[0131] 100 μl of a reaction mixture [20 mM HEPES-potassium hydroxide (pH 7.8), 0.01% bovine serum albumin (Takara Shuzo), 1% dimethyl sulfoxide, 4 mM magnesium acetate, 20 μg/ml poly(dT) (Amersham Pharmacia Biotech), 30 μg/ml poly(rA) (Amersham Pharmacia Biotech)] which had been incubated at 40°C was added to 1 μl of the Bca RNase HIII preparation. The mixture was reacted at 40°C for 10 minutes. The reaction was terminated by adding 10 μl of 0.5 M EDTA (pH 8.0). Absorbance at 260 nm was then measured.

[0132] As a result, an RNase H activity was observed for the Bca RNase HIII preparation.

Example 4: Cloning of Pyrococcus furiosus RNase HII gene

(1) Preparation of genomic DNA from Pyrococcus furiosus

[0133] 2 L of a medium containing 1% Tryptone (Difco Laboratories), 0.5% yeast extract (Difco Laboratories), 1% soluble starch (Nacalai Tesque), 3.5% Jamarine S Solid (Jamarine Laboratory), 0.5% Jamarine S Liquid (Jamarine Laboratory), 0.003% $MgSO_4$, 0.001% NaCl, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0001% $COSO_4$, 0.0001% $CaCl_2 \cdot 7H_2O$, 0.0001% $ZnSO_4$, 0.1 ppm $CuSO_4 \cdot 5H_2O$, 0.1 ppm $KAl(SO_4)_2$, 0.1 ppm H_3BO_4, 0.1 ppm $Na_2MoO_4 \cdot 2H_2O$ and 0.25 ppm $NiCl_2 \cdot 6H_2O$ was placed in a 2-L medium bottle, sterilized at 120°C for 20 minutes, bubbled with nitrogen gas to remove dissolved oxygen, then Pyrococcus furiosus (purchased from Deutsche Sammlung von Mikroorganismen; DSM3638) was inoculated into the medium and cultured at 95°C for 16 hours without shaking. After cultivation, cells were collected by centrifugation.

[0134] The resulting cells were then suspended in 4 ml of 25% sucrose, 50 mM tris-HCl (pH 8.0). 0.4 ml of 10 mg/ml lysozyme chloride (Nacalai Tesque) in water was added thereto. The mixture was reacted at 20°C for 1 hour. After reaction, 24 ml of a mixture containing 150 mM NaCl, 1 mM EDTA and 20 mM tris-HCl (pH 8.0), 0.2 ml of 20 mg/ml proteinase K (Takara Shuzo) and 2 ml of 10% aqueous solution of sodium lauryl sulfate were added to the reaction mixture. The mixture was incubated at 37°C for 1 hour.

[0135] After reaction, the mixture was subjected to phenol-chloroform extraction followed by ethanol precipitation to prepare about 1 mg of genomic DNA.

(2) Cloning of RNase HII gene

[0136] The entire genomic sequence of Pyrococcus horikoshii was published [Kawarabayashi, Y. et al., DNA Research, 5:55-76 (1998)]. The existence of one gene encoding a homologue of RNase HII (PH1650) in the genome was known (SEQ ID NO:19, the home page of National Institute of Technology and Evaluation: http://www.nite.go.jp/).

[0137] Homology between the PH1650 gene (SEQ ID NO:19) and the partially published genomic sequence of Pyrococcus furiosus (the home page of University of Utah, Utah Genome Center: http://www.genome.utah.edu/sequence.html) was searched. As a result, a highly homologous sequence was found.

[0138] Primers 1650Nde (SEQ ID NO:20) and 1650Bam (SEQ ID NO:21) were synthesized on the basis of the homologous sequence.

[0139] A PCR was carried out in a volume of 100 μl using 200 ng of the Pyrococcus furiosus DNA obtained in Example 4-(1) as a template, and 20 pmol each of 1650Nde and 1650Bam as primers. TaKaRa Ex Taq (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. An amplified DNA fragment of about 0.7 kb was digested with NdeI and BamHI (both from Takara Shuzo). The resulting DNA fragment was inserted between the NdeI site and the BamHI site in a plasmid vector pET3a (Novagen) to make a plasmid pPFU220.

(3) Determination of nucleotide sequence of DNA fragment containing RNase HII gene

[0140] The nucleotide sequence of the DNA fragment inserted into pPFU220 obtained in Example 4-(2) was determined according to a dideoxy method.

[0141] Analysis of the determined nucleotide sequence revealed the existence of an open reading frame presumably encoding RNase HII. The nucleotide sequence of the open reading frame is shown in SEQ ID NO:22. The amino acid sequence of RNase HII deduced from the nucleotide sequence is shown in SEQ ID NO:23.

[0142] Escherichia coli JM109 transformed with the plasmid pPFU220 is designated and indicated as Escherichia coli JM109/pPFU220, and deposited on September 5, 2000 (date of original deposit) at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566, Japan under accession number FERM BP-7654.

(4) Preparation of purified RNase HII preparation

[0143] Escherichia coli HMS174(DE3) (Novagen) was transformed with pPFU220 obtained in Example 4-(2). The resulting Escherichia coli HMS174(DE3) harboring pPFU220 was inoculated into 2 L of LB medium containing 100 μg/ml of ampicillin and cultured with shaking at 37°C for 16 hours. After cultivation, cells collected by centrifugation were suspended in 66.0 ml of a sonication buffer [50 mM tris-HCl (pH 8.0), 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride] and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12,000 rpm for 10 minutes was heated at 80°C for 15 minutes. It was then centrifuged again at 12,000 rpm for 10 minutes to collect a supernatant. Thus, 61.5 ml of a heated supernatant was obtained.
[0144] The heated supernatant was subjected to RESOURSE Q column (Amersham Pharmacia Biotech) equilibrated with Buffer A [50 mM tris-HCl (pH 8.0), 1 mM EDTA] and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HII flowed through the RESOURSE Q column.
[0145] 60.0 ml of the flow-through RNase HII fraction was subjected to RESOURSE S column (Amersham Pharmacia Biotech) equilibrated with Buffer A and eluted with a linear gradient of 0 to 500 mM NaCl using FPLC system. A fraction containing RNase HII eluted with about 150 mM NaCl was obtained.
[0146] 2.0 ml of the RNase HII fraction was concentrated by ultrafiltrationusing Centricon-10 (Millipore). 250 μl of the concentrate was subjected to Superdex 200 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 50 mM tris-HCl (pH 8.0) containing 100 mM NaCl and 0.1 mM EDTA and eluted with the same buffer. As a result, RNase HII was eluted at a position corresponding to a molecular weight of 17 kilodalton. This molecular weight corresponds to that of the RNase HII in the monomeric form.
[0147] The thus eluted RNase HII was used as Pfu RNase HII preparation.
[0148] The enzymatic activity of the thus obtained Pfu RNase HII preparation was measured as described in Example 3-(5). As a result, an RNase H activity was observed for the Pfu RNase HII preparation.

Example 5: Cloning of Thermotoga maritima RNase HII gene

(1) Preparation of genomic DNA from Thermotoga maritima

[0149] 2 L of a medium containing 1% Tryptone, 0.5% yeast extract, 1% soluble starch, 3.5% Jamarine S Solid, 0.5% Jamarine S Liquid, 0.003% $MgSO_4$, 0.001% NaCl, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0001% $CoSO_4$, 0.0001% $CaCl_2 \cdot 7H_2O$, 0.0001% $ZnSO_4$, 0.1 ppm $CuSO_4 \cdot 5H_2O$, 0.1 ppm $KAl(SO_4)_2$, 0.1 ppm H_3BO_3, 0.1 ppm $Na_2MoO_4 \cdot 2H_2O$ and 0.25 ppm $NiCl_2 \cdot 6H_2O$ was placed in a 2-L medium bottle, sterilized at 120°C for 20 minutes, bubbled with nitrogen gas to remove dissolved oxygen, then Thermotoga maritima (purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSM3109) was inoculated into the medium and cultured at 85°C for 16 hours without shaking.
[0150] Cells collected by centrifugation from 300 ml of the culture were then suspended in 3 ml of TE buffer [10 mM tris-HCl (pH 7.5), 1 mM EDTA]. 150 μl of 10% aqueous solution of sodium lauryl sulfate (Nacalai Tesque) and 15 μl of 20 mg/ml proteinase K (Takara Shuzo) were added thereto. The mixture was incubated at 37°C for 1 hour.
[0151] After reaction, 0.5 ml of 5 M NaCl was added to the mixture. After thoroughly mixing, 0.4 ml of a CTAB-NaCl solution [10% cetyltrimethylammonium bromide (Nacalai Tesque), 0.7 M NaCl] was added to the mixture. After thoroughly mixing, the mixture was incubated at 65°C for 10 minutes. 1.5 ml of a mixture of chloroform/isoamyl alcohol (24:1, v/v) was added thereto. The mixture was gently mixed for 10 minutes and centrifuged at 20,000 x g for 5 minutes. After centrifugation, an equal volume of a mixture of phenol saturated with 100 mM tris-HCl (pH 8.0)/chloroform/isoamyl alcohol (25:24:1, v/v) was added to the resulting supernatant. The mixture was gently mixed for 10 minutes and then centrifuged at 20,000 x g for 5 minutes. After centrifugation, a 0.6 volume of 2-propanol was added to the supernatant. The precipitate obtained by centrifugation at 10,000 x g for 5 minutes was washed with 70% ethanol in water, air-dried and then dissolved in 200 μl of TE to obtain a genomic DNA solution.

(2) Cloning of RNase HII gene

[0152] Oligonucleotides 915-F1, 915-F2, 915-R1 and 915-R2 represented by SEQ ID NOS:24 to 27 were synthesized on the basis of the nucleotide sequence of a portion that had been identified as an RNase HII gene in the nucleotide sequence of the genomic DNA of Thermotoga maritima (http://www.tigr.org/tdb/CMR/btm/htmls/SplashPage.html) in order to obtain an amplified DNA fragment containing an RNase HII gene by carrying out a PCR using the Thermotoga maritima genomic DNA as a template.
[0153] PCRs were carried out using the Thermotoga maritima genomic DNA as prepared in Example 5-(1) as a template, and 915-F1 and 915-R1, 915-F1 and 915-R2, 915-F2 and 915-R1, or 915-F2 and 915-R2 as a primer pair. TaKaRa Ex Taq (Takara Shuzo) was used as a DNA polymerase for the PCRs according to the attached protocol. The PCRs were carried out as follows: 25 cycles of 95°C for 0.5 minute, 55°C for 0.5 minute and 72°C for 1.5 minute. After

reactions, the respective PCR products were subjected to agarose gel electrophoresis to extract and purify amplified DNA fragments of about 0.7 kb.

[0154] The DNAs amplified using a primer pair 915-F1 and 915-R1 or 915-F1 and 915-R2 were digested with HindIII and XbaI (both from Takara Shuzo) and ligated with pUC19 (Takara Shuzo) digested with HindIII and XbaI using T4 DNA ligase (Takara Shuzo). The ligation mixture was used to transform Escherichia coli JM109.

[0155] The resulting transformants were cultured to prepare plasmid DNAs into which the about 0.7-kb DNA fragments were inserted. As a result, plasmids No. 1 and No. 2 having DNA fragments amplified using 915-F1 and 915-R1, and plasmids No. 3 and No. 4 having DNAs amplified using 915-F1 and 915-R2 were obtained.

[0156] In addition, the DNAs amplified using a primer pair 915-F2 and 915-R1 or 915-F2 and 915-R2 were doubly digested with NcoI (Takara Shuzo) and XbaI and ligated with pTV119N (Takara Shuzo) doubly digested with NcoI and XbaI using T4 DNA ligase. The ligation mixture was used to transform Escherichia coli JM109.

[0157] The resulting transformants were cultured to prepare plasmid DNAs into which the about 0.7-kb DNA fragments were inserted. As a result, plasmids No. 5 and No. 6 having DNA fragments amplified using 915-F2 and 915-R1, and a plasmid No. 7 having a DNA amplified using 915-F2 and 915-R2 were obtained.

[0158] Escherichia coli JM109 transformed with the plasmid No. 7 is designated and indicated as Escherichia coli JM109/pTM-RNH, and deposited on September 5, 2000 (date of original deposit) at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566, Japan under accession number FERM BP-7652.

(3) Expression of Thermotoga maritima RNase HII gene

[0159] Escherichia coli JM109 transformed with one of the plasmids No. 1 to 7 or pUC19 was inoculated into 5 ml of LB medium (10 g/L Tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.2) containing 100 μg/ml of ampicillin and cultured with shaking at 37°C. When the absorbance at 660 nm reached 0.5, isopropyl-β-D-thiogalactopyranoside was added thereto to a final concentration of 1 mM and the cells were cultured overnight. After cultivation, cells collected by centrifugation were suspended in 1 ml of TE buffer and sonicated. The sonicated suspension was heated at 80°C for 10 minute. A supernatant obtained by centrifugation was used as a crude cell extract.

[0160] Absorbance was measured using the crude cell extract as described in Example 2-(5).

[0161] As a result, when reactions were carried out in the presence of $MnCl_2$, the absorbance at 260 nm from each of the reactions in which crude cell extracts prepared from Escherichia coli JM109 harboring the plasmid No. 3, 5, 6 or 7 were used was clearly higher than that from a reaction in which a crude extract prepared from Escherichia coli JM109 harboring pUC19 was used. Thus, it was demonstrated that the plasmids No. 3, 5, 6 and 7 contained RNase HII genes and that Escherichia coli harboring one of these plasmids expressed an RNase H activity.

[0162] The nucleotide sequences of the DNA fragments inserted into the plasmids which were demonstrated to express RNase H activities in Escherichia coli were determined. Analysis of the determined nucleotide sequences revealed an open reading frame presumably encoding RNase HII. The nucleotide sequence of the open reading frame is shown in SEQ ID NO:58. The amino acid sequence of RNase HII deduced from the nucleotide sequence is shown in SEQ ID NO:59. Then, it was found that one base substitution that was presumably generated upon the PCR was observed in the nucleotide sequence of the DNA fragment inserted in the plasmid No. 7, resulting in the change in the encoded amino acid residue.

(4) Preparation of purified RNase HII preparation

[0163] Escherichia coli JM109 was transformed with the plasmid No. 7 (pTM-RNH) obtained in Example 5-(2). The resulting Escherichia coli JM109 harboring pTM-RNH was inoculated into 1 L of LB medium containing 100 μg/ml of ampicillin and cultured with shaking at 37°C for 16 hours. After cultivation, cells collected by centrifugation were suspended in 31.0 ml of a sonication buffer [50 mM tris-HCl (pH 8.0), 2 mM 2-mercaptoethanol, 10% glycerol, 2 mM phenylmethanesulfonyl fluoride] and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12,000 rpm for 10 minutes was heated at 70°C for 15 minutes. It was then centrifuged again at 12,000 rpm for 10 minutes to collect a supernatant. Thus, 32.0 ml of a heated supernatant was obtained.

[0164] The heated supernatant was subjected to RESOURSE Q column (Amersham Pharmacia Biotech) equilibrated with Buffer C [50 mM tris-HCl (pH 8.0), 2 mM 2-mercaptoethanol, 10% glycerol] and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HII flowed through the RESOURSE Q column.

[0165] 32.5 ml of the flow-through RNase HII fraction was subjected to RESOURSE S column (Amersham Pharmacia Biotech) equilibrated with Buffer C and eluted with a linear gradient of 0 to 500 mM NaCl using FPLC system. A fraction containing RNase HII eluted with about 240 mM NaCl was obtained.

[0166] 2.0 ml of the RNase HII fraction was subjected to PD-10 column (Amersham Pharmacia Biotech) equilibrated with Buffer C containing 50 mM NaCl. 3.5 ml of the resulting eluate was subjected to HiTrap-heparin column (Amersham

Pharmacia Biotech) equilibrated with Buffer C containing 50 mM NaCl and eluted with a linear gradient of 50 to 550 mM NaCl using FPLC system. As a result, a fraction containing RNase HII eluted with about 295 mM NaCl was obtained.
[0167] The thus eluted RNase HII was used as Tma RNase HII preparation.
[0168] The enzymatic activity of the thus obtained Tma RNase HII preparation was measured as described in Example 2-(6). As a result, an RNase H activity was observed for the Tma RNase HII preparation.

Example 6: Cloning of RNase HII gene from Pyrococcus horikoshii

(1) Preparation of genomic DNA from Pyrococcus horikoshii

[0169] 2 L of a medium containing 1% Tryptone (Difco Laboratories), 0.5% yeast extract (Difco Laboratories), 1% soluble starch (Nacalai Tesque), 3.5% Jamarine S Solid (Jamarine Laboratory), 0.5% Jamarine S Liquid (Jamarine Laboratory), 0.003% $MgSO_4$, 0.001% NaCl, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0001% $CoSO_4$, 0.0001% $CaCl_2 \cdot 7H_2O$, 0.0001% $ZnSO_4$, 0.1 ppm $CuSO_4 \cdot 5H_2O$, 0.1 ppm $KAl(SO_4)_2$, 0.1 ppm H_3BO_4, 0.1 ppm $Na_2MoO_4 \cdot 2H_2O$ and 0.25 ppm $NiCl_2 \cdot 6H_2O$ was placed in a 2-L medium bottle, sterilized at 120°C for 20 minutes, bubbled with nitrogen gas to remove dissolved oxygen, then Pyrococcus horikoshii OT3 (purchased from the Institute of Physical and Chemical Research (RIKEN); JCM9974) was inoculated into the medium and cultured at 95°C for 16 hours without shaking. After cultivation, cells were collected by centrifugation.
[0170] The cells were then suspended in 4 ml of 25% sucrose, 50 mM tris-HCl (pH 8.0). 0.4 ml of 10 mg/ml lysozyme chloride (Nacalai Tesque) in water was added thereto. The mixture was reacted at 20°C for 1 hour. After reaction, 24 ml of a mixture containing 150 mM NaCl, 1 mM EDTA and 20 mM tris-HCl (pH 8.0), 0.2 ml of 20 mg/ml proteinase K (Takara Shuzo) and 2 ml of 10% aqueous solution of sodium lauryl sulfate were added to the reaction mixture. The mixture was incubated at 37°C for 1 hour.
[0171] After reaction, the mixture was subjected to phenol-chloroform extraction followed by ethanol precipitation to prepare about 1 mg of genomic DNA.

(2) Cloning of RNase HII gene

[0172] The entire genomic sequence of the Pyrococcus horikoshii has been published [DNA Research, 5:55-76 (1998)]. The existence of one gene encoding a homologue of RNase HII (PH1650) was known (SEQ ID NO:28, the home page of National Institute of Technology and Evaluation: http://www.nite.go.jp/).
[0173] Primers PhoNde (SEQ ID NO:29) and PhoBam (SEQ ID NO:30) were synthesized on the basis of the sequence of the PH1650 gene (SEQ ID NO:28).
[0174] A PCR was carried out using 100 ng of the Pyrococcus horikoshii DNA prepared in Example 6-(1) as a template, and 20 pmol each of PhoNde and PhoBam as primers in a volume of 100 µl. TaKaRa Ex Taq (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. An amplified DNA fragment of about 0.7 kb was digested with NdeI and BamHI (both from Takara Shuzo). Then, a plasmid pPHO238 was constructed by incorporating the resulting DNA fragment between NdeI and BamHI sites in a plasmid vector pET3a (Novagen).

(3) Determination of nucleotide sequence of DNA fragment containing RNase HII gene

[0175] The nucleotide sequence of the DNA fragment inserted into pPHO238 obtained in Example 6-(2) was determined according to a dideoxy method.
[0176] Analysis of the determined nucleotide sequence revealed an open reading frame presumably encoding RNase HII. The nucleotide sequence of the open reading frame is shown in SEQ ID NO:31. The amino acid sequence of RNase HII deduced from the nucleotide sequence is shown in SEQ ID NO:32.
[0177] Escherichia coli JM109 transformed with the plasmid pPHO238 is designated and indicated as Escherichia coli JM109/pPHO238, and deposited on February 22, 2001 (date of original deposit) at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566, Japan under accession number FERM BP-7692.

(4) Preparation of purified RNase HII preparation

[0178] Escherichia coli HMS174(DE3) (Novagen) was transformed with pPHO238 obtained in Example 6-(2). The resulting Escherichia coli HMS174(DE3) harboring pPHO238 was inoculated into 1 L of LB medium containing 100 µg/ml of ampicillin and cultured with shaking at 37°C for 16 hours. After cultivation, cells collected by centrifugation were suspended in 34.3 ml of a sonication buffer [50 mM tris-HCl (pH 8.0), 1 mM EDTA, 2 mM phenylmethanesulfonyl

fluoride] and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12,000 rpm for 10 minutes was heated at 80°C for 15 minutes. It was then centrifuged again at 12,000 rpm for 10 minutes to collect a supernatant. Thus, 33.5 ml of a heated supernatant was obtained.

[0179] The heated supernatant was subjected to RESOURSE Q column (Amersham Pharmacia Biotech) equilibrated with Buffer A [50 mM tris-HCl (pH 8.0), 1 mM EDTA] and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HII flowed through the RESOURSE Q column.

[0180] 35.0 ml of the flow-through RNase HII fraction was dialyzed against 2 L of Buffer B (50 mM tris-HCl (pH 7.0), 1 mM EDTA) for 2 hours. The dialysis was repeated two more times. 34.5 ml of the dialyzed enzyme solution was subjected to RESOURSE S column (Amersham Pharmacia Biotech) equilibrated with Buffer B and eluted with a linear gradient of 0 to 500 mM NaCl using FPLC system. A fraction containing RNase HII eluted with about 155 mM NaCl was obtained.

[0181] Buffer B was added to 4.0 ml of the fraction to make the NaCl concentration to 50 mM. The mixture was subjected to HiTrap-heparin column (Amersham Pharmacia Biotech) equilibrated with Buffer B containing 50 mM NaCl and eluted with a linear gradient of 50 to 550 mM NaCl using FPLC system. As a result, a fraction containing RNase HII eluted with about 160 mM NaCl was obtained.

[0182] 6.9 ml of the RNase HII fraction was concentrated by ultrafiltration using Centricon-10 (Millipore). Two portions each separated from 250 µl of the concentrate were subjected to Superose 6 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 50 mM tris-HCl (pH 7.0) containing 100 mM NaCl and 0.1 mM EDTA and eluted with the same buffer. As a result, RNase HII was eluted at a position corresponding to a molecular weight of 24.5 kilodalton. This molecular weight corresponds to that of the RNase HII in the monomeric form.

[0183] The RNase HII eluted as described above was used as Pho RNase HII preparation.

[0184] The enzymatic activity of the thus obtained Pho RNase HII preparation was measured as described in Example 3-(5) . As a result, an RNase H activity was observed for the Pho RNase HII preparation.

Example 7: Cloning of RNase HII gene from Archaeoglobus fulgidus

(1) Preparation of genomic DNA from Archaeoglobus fulgidus

[0185] Cells of Archaeoglobus fulgidus (purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSM4139) collected from 8 ml of a culture was suspended in 100 µl of 25% sucrose, 50 mM tris-HCl (pH 8.0). 20 µl of 0.5 M EDTA and 10 µl of 10 mg/ml lysozyme chloride (Nacalai Tesque) in water was added thereto. The mixture was reacted at 20°C for 1 hour. After reaction, 800 µl of a mixture containing 150 mM NaCl, 1 mM EDTA and 20 mM tris-HCl (pH 8.0), 10 µl of 20 mg/ml proteinase K (Takara Shuzo) and 50 µl of 10% aqueous solution of sodium lauryl sulfate were added to the reaction mixture. The mixture was incubated at 37°C for 1 hour. After reaction, the mixture was subjected to phenol-chloroform extraction, ethanol precipitation and air-drying, and then dissolved in 50 µl of TE to obtain a genomic DNA solution.

(2) Cloning of RNase HII gene

[0186] The entire genomic sequence of the Archaeoglobus fulgidus has been published [Klenk, HP et al., Nature, 390:364-370 (1997)]. The existence of one gene encoding a homologue of RNase HII (AF0621) was known (SEQ ID NO:33, http://www.tigr.org/tdb/CMR/btm/htmls/SplashPage.htlm).

[0187] Primers AfuNde (SEQ ID NO:34) and AfuBam (SEQ ID NO:35) were synthesized on the basis of the sequence of the AF0621 gene (SEQ ID NO:33).

[0188] A PCR was carried out using 30 ng of the Archaeoglobus fulgidus genomic DNA prepared in Example 7-(1) as a template, and 20 pmol each of AfuNde and AfuBam as primers in a volume of 100 µl. Pyrobest DNA polymerase (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. An amplified DNA fragment of about 0.6 kb was digested with NdeI and BamHI (both from Takara Shuzo). Then, a plasmid pAFU204 was constructed by incorporating the resulting DNA fragment between NdeI and BamHI sites in a plasmid vector pTV119Nd (a plasmid in which the NcoI site in pTV119N is converted into a NdeI site).

(3) Determination of nucleotide sequence of DNA fragment containing RNase HII gene

[0189] The nucleotide sequence of the DNA fragment inserted into pAFU204 obtained in Example 7-(2) was determined according to a dideoxy method.

[0190] Analysis of the determined nucleotide sequence revealed an open reading frame presumably encoding RNase HII. The nucleotide sequence of the open reading frame is shown in SEQ ID NO:36. The amino acid sequence of

RNase HII deduced from the nucleotide sequence is shown in SEQ ID NO:37.

[0191] Escherichia coli JM109 transformed with the plasmid pAFU204 is designated and indicated as Escherichia coli JM109/pAFU204, and deposited on February 22, 2001 (date of original deposit) at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566, Japan under accession number FERM BP-7691.

(4) Preparation of purified RNase HII preparation

[0192] Escherichia coli JM109 was transformed with pAFU204 obtained in Example 7-(2). The resulting Escherichia coli JM109 harboring pAFU204 was inoculated into 2 L of LB medium containing 100 μg/ml of ampicillin and cultured with shaking at 37°C for 16 hours. After cultivation, cells collected by centrifugation were suspended in 37.1 ml of a sonication buffer [50 mM tris-HCl (pH 8.0), 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride] and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12,000 rpm for 10 minutes was heated at 70°C for 15 minutes. It was then centrifuged again at 12,000 rpm for 10 minutes to collect a supernatant. Thus, 40.3 ml of a heated supernatant was obtained.

[0193] The heated supernatant was subjected to RESOURSE Q column (Amersham Pharmacia Biotech) equilibrated with Buffer A [50 mM tris-HCl (pH 8.0), 1 mM EDTA] and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HII flowed through the RESOURSE Q column.

[0194] The flow-through RNase HII fraction was subjected to RESOURSE S column (Amersham Pharmacia Biotech) equilibrated with Buffer A and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HII flowed through the RESOURSE S column.

[0195] 40.0 ml of the flow-through RNase HII fraction was dialyzed against 2 L of Buffer B (50 mM tris-HCl (pH 7.0), 1 mM EDTA) containing 50 mM NaCl for 2 hours. The dialysis was repeated two more times. 40.2 ml of the dialyzed enzyme solution was subjected to HiTrap-heparin column (Amersham Pharmacia Biotech) equilibrated with Buffer B containing 50 mM NaCl and eluted with a linear gradient of 50 to 550 mM NaCl using FPLC system. As a result, a fraction containing RNase HII eluted with about 240 mM NaCl was obtained.

[0196] 7.8 ml of the RNase HII fraction was concentrated by ultrafiltration using Centricon-10 (Millipore). Four portions each separated from about 600 μl of the concentrate were subjected to Superose 6 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 50 mM tris-HCl (pH 7.0) containing 100 mM NaCl and 0.1 mM EDTA and eluted with the same buffer. As a result, RNase HII was eluted at a position corresponding to a molecular weight of 30.0 kilodalton. This molecular weight corresponds to that of the RNase HII in the monomeric form.

[0197] The RNase HII eluted as described above was used as Afu RNase HII preparation.

[0198] The enzymatic activity of the thus obtained Afu RNase HII preparation was measured as described in Example 3-(5). As a result, an RNase H activity was observed for the Afu RNase HII preparation.

Example 8: Cloning of Thermococcus litoralis RNase HII gene

(1) Preparation of genomic DNA from Thermococcus litoralis

[0199] Thermococcus litoralis (purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSM5473) cells were collected from 11 ml of culture. The cells were suspended in 500 μl of 25% sucrose and 50 mM tris-HCl (pH 8.0). 100 μl of 0.5 M EDTA and 50 μl of 10 mg/ml lysozyme chloride (Nacalai Tesque) in water was added thereto. The mixture was reacted at 20°C for 1 hour. After reaction, 4 ml of a mixture containing 150 mM NaCl, 1 mM EDTA and 20 mM tris-HCl (pH 8.0), 50 μl of 20 mg/ml proteinase K (Takara Shuzo) and 250 μl of a 10% aqueous solution of sodium lauryl sulfate were added to the reaction mixture. The mixture was incubated at 37°C for 1 hour. After reaction, the reaction was subjected to phenol-chloroform extraction and ethanol precipitation, air-dried and then dissolved in 100 μl of TE buffer to obtain a genomic DNA solution.

(2) Cloning of middle portion of RNase HII gene

[0200] Oligonucleotides RN-F1 and RN-R0 represented by SEQ ID NOS:38 and 39 were synthesized on the basis of portions conserved among amino acid sequences of various thermostable RNase HIIs.

[0201] A PCR was carried out in a volume of 100 ul using 5 μl of the Thermococcus litoralis genomic DNA solution as prepared in Example 8-(1) as a template, and 100 pmol each of RN-F1 and RN-R0 as primers. TaKaRa Taq (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 50 cycles of 94°C for 30 seconds, 45°C for 30 seconds and 72°C for 1 minute. After reaction, Microcon-100 (Takara Shuzo) was used to remove primers from the reaction mixture and to concentrate the reaction mixture.

(3) Cloning of upstream and downstream portions of RNase HII gene

[0202] The nucleotide sequence of the inserted fragment of about 0.5 kb, TliF1R0, obtained in Example 8-(2) was determined. A specific oligonucleotide TliRN-1 (SEQ ID NO:40) for cloning the upstream portion and a specific oligonucleotide TliRN-2 (SEQ ID NO:41) for cloning the downstream portion were synthesized on the basis of the determined nucleotide sequence. In addition, 48 primers as shown in Table 1 were synthesized. The tag sequence in Table 1 is shown in SEQ ID NO:60.

Table 1

5'-tag sequence-NN-SSSSSSS-3'
(N: mixture of G, A, T and C; S represents the nucleotide sequence below)

No.	Nucleotide sequence	No.	Nucleotide sequence	No.	Nucleotide sequence
1	ggagcag	19	gtaacgg	37	gcgcaag
2	ggcaaag	20	gtaagcg	38	gcgcttg
3	ggcaacg	21	gtacacg	39	gcggacg
4	ggcacag	22	gtagacg	40	gcgtaag
5	ggcattg	23	gtagcgg	41	gctacgg
6	ggccaag	24	gtcaacg	42	gctcacg
7	ggccttg	25	gcaccag	43	gctccag
8	ggctaag	26	gcagacg	44	gcttgcg
9	ggctacg	27	gcagcag	45	gcttggg
10	ggctcag	28	gcatggg	46	ggacacg
11	ggctttg	29	gccaaag	47	ggaccag
12	gggacag	30	gccacag	48	ggagacg
13	gggcaag	31	gccattg		
14	gggcttg	32	gcccaag		
15	gggtacg	33	gcccttg		
16	ggtaacg	34	gcctacg		
17	ggtacgg	35	gcctcag		
18	ggtagcg	36	gcctttg		

[0203] PCRs were carried out in reaction mixtures containing 1 μl of the Thermococcus litoralis genomic DNA solution prepared in Example 8-(1) as a template, a combination of 20 pmol of TliRN-1 or 20 pmol of TliRN-2 and 20 pmol of one of the 48 primers listed in Table 1, 20 mM tris-acetate (pH 8.5), 50 mM potassium acetate, 3 mM magnesium acetate, 0.01% BSA, 30 μM each of dNTPs and 2.5 units of TaKaRa Ex Taq DNA polymerase (Takara Shuzo). PCRs were carried out as follows: incubation at 94°C for 3 minutes; and 40 cycles of 98°C for 10 seconds, 50°C for 10 seconds and 72°C for 40 seconds. A portion of each PCR product was subjected to agarose gel electrophoresis. Microcon-100 (Takara Shuzo) was used to remove primers from reaction mixtures that resulted in single bands and to concentrate the reaction mixtures. The concentrates were subjected to direct sequencing to screen for fragments containing the upstream or downstream portions of the RNase HII. As a result, it was shown that an about 450-bp PCR-amplified fragment TliN7 contained the upstream portion of the RNase HII gene and an about 600-bp PCR-amplified fragment TliC25 and an about 400-bp PCR-amplified fragment TliC26 contained the downstream portion of the RNase HII gene, respectively.

(4) Cloning of entire RNase HII gene

[0204] The nucleotide sequence of a gene containing Tli RNase HII is shown in SEQ ID NO:42. The amino acid sequence of RNase HII deduced from the nucleotide sequence is shown in SEQ ID NO:43. Primers TliNde (SEQ ID NO:44) and TliBam (SEQ ID NO:45) were synthesized on the basis of the nucleotide sequence.

[0205] A PCR was carried out in a volume of 100 ul using 1 ul of the Thermococcus litoralis genomic DNA solution as prepared in Example 8-(1) as a template, and 20 pmol each of TliNde and TliBam as primers. Ex Taq DNA polymerase (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. An amplified DNA fragment of about 0.7 kb was digested with NdeI and BamHI (both from Takara Shuzo). Then, plasmids pTLI223Nd and pTLI204 were constructed by incorporating the resulting DNA fragment between NdeI and BamHI sites in a plasmid vector pTV119Nd (a plasmid in which the NcoI site in pTV119N is converted into a NdeI site) or pET3a (Novagen), respectively.

(5) Determination of nucleotide sequence of DNA fragment containing RNase HII gene

[0206] The nucleotide sequences of the DNA fragments inserted into pTLI223Nd and pTLI204 obtained in Example 8-(4) were determined according to a dideoxy method.

[0207] Analyses of the determined nucleotide sequences revealed the existence of open reading frames each presumably encoding RNase HII. The nucleotide sequence of the open reading frame in pTLI204 is shown in SEQ ID NO: 46. The amino acid sequence of RNase HII deduced from the nucleotide sequence is shown in SEQ ID NO:47. "T" at position 484 in the nucleotide sequence of the open reading frame in pTLI204 was replaced by "C" in the nucleotide sequence of the open reading frame in pTLI223Nd. In the amino acid sequence, phenylalanine at position 162 was replaced by leucine.

[0208] Escherichia coli HMS174(DE3) transformed with the plasmid pTLI204 is designated and indicated as Escherichia coli HMS174(DE3)/pTLI204, and deposited on February 22, 2001 (date of original deposit) at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566, Japan under accession number FERM BP-7693.

(6) Expression of Thermococcus litoralis RNase HII gene

[0209] Escherichia coli JM109 transformed with pTLI223Nd was inoculated into 10 ml of LB medium containing 100 µg/ml of ampicillin and 1 mM IPTG and cultured with shaking at 37°C overnight. After cultivation, cells collected by centrifugation were suspended in 196 µl of Buffer A and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12,000 rpm for 10 minutes was heated at 70°C for 10 minutes and then centrifuged again at 12,000 rpm for 10 minutes to collect a supernatant as a heated supernatant. Similarly, Escherichia coli HMS174(DE3) transformed with pTLI204 was inoculated into 10 ml of LB medium containing 100 µg/ml of ampicillin and cultured with shaking at 37°C overnight. After cultivation, cells collected by centrifugation were processed according to the procedure as described above to obtain a heated supernatant.

[0210] The enzymatic activities were measured for the heated supernatants as described in Example 3-(5). As a result, RNase H activities were observed for both transformants. Thus, the activity of the polypeptide of the present invention was confirmed in spite of substitution in the nucleotide sequence or the amino acid sequence.

(7) Preparation of purified RNase HII preparation

[0211] Escherichia coli JM109 transformed with pTLI223Nd obtained in Example 8-(4) was inoculated into 2 L of LB medium containing 100 µg/ml of ampicillin and cultured with shaking at 37°C for 16 hours. After cultivation, cells collected by centrifugation were suspended in 38.7 ml of a sonication buffer [50 mM tris-HCl (pH 8.0), 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride] and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12,000 rpm for 10 minutes was heated at 70°C for 15 minutes. It was then centrifuged again at 12,000 rpm for 20 minutes to collect a supernatant. Thus, 37.2 ml of a heated supernatant was obtained.

[0212] The heated supernatant was subjected to RESOURSE Q column (Amersham Pharmacia Biotech) equilibrated with Buffer A [50 mM tris-HCl (pH 8.0), 1 mM EDTA] and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HII flowed through the RESOURSE Q column.

[0213] The sample was subjected to RESOURSE S column (Amersham Pharmacia Biotech) equilibrated with Buffer A and eluted with a linear gradient of 0 to 500 mM NaCl using FPLC system (Amersham Pharmacia Biotech). A fraction containing RNase HII eluted with about 220 mM NaCl was obtained.

[0214] A mixture containing 3 ml of the RNase HII fraction and Buffer A added to make the NaCl concentration to 50 mM was subjected to HiTrap-heparin column (Amersham Pharmacia Biotech) equilibrated with Buffer A containing 50 mM NaCl and eluted with a linear gradient of 50 to 550 mM NaCl using FPLC system. As a result, a fraction containing RNase HII eluted with about 320 mM NaCl was obtained.

[0215] 6 ml of the RNase HII fraction was concentrated by ultrafiltration using Centricon-10 (Millipore). About 198 µl of the concentrate was subjected to Superose 6 gel filtration column (Amersham Pharmacia Biotech) equilibrated with

50 mM tris-HCl (pH 8.0) containing 100 mM NaCl and 0.1 mM EDTA and eluted with the same buffer. As a result, RNase HII was eluted at a position corresponding to a molecular weight of 26.5 kilodalton. This molecular weight corresponds to that of the RNase HII in the monomeric form. The thus eluted RNase HII was used as Tli RNase HII preparation.

[0216] The enzymatic activity of the thus obtained Tli RNase HII preparation was measured as described in Example 3-(5). As a result, an RNase H activity was observed for the Tli RNase HII preparation.

Example 9: Cloning of Thermococcus celer RNase HII gene

(1) Preparation of genomic DNA from Thermococcus celer

[0217] Thermococcus celer (purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSM2476) cells were collected from 11 ml of culture. A genomic DNA solution was obtained as described in Example 8-(1)

(2) Cloning of middle portion of RNase HII gene

[0218] Oligonucleotides RN-F1 and RN-R0 represented by SEQ ID NOS:48 and 49 were synthesized on the basis of portions conserved among amino acid sequences of various thermostable RNase HIIs.

[0219] A PCR was carried out in a volume of 100 μl using 5 μl of the Thermococcus celer genomic DNA solution as prepared in Example 9-(1) as a template, and 100 pmol each of RN-F1 and RN-R0 as primers. TaKaRa Taq (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 50 cycles of 94°C for 30 seconds, 45°C for 30 seconds and 72°C for 1 minute. After reaction, an about 500-bp DNA fragment obtained by amplification was blunt-ended using T4 DNA polymerase (Takara Shuzo) and then subjected to agarose gel electrophoresis to recover an amplified DNA fragment of about 500 bp. The about 500-bp DNA fragment was ligated with pUC119 (Takara Shuzo) digested with SmaI (Takara Shuzo) using T4 DNA ligase (Takara Shuzo). The ligation mixture was used to transform Escherichia coli JM109.

[0220] The resulting transformants were cultured to obtain a plasmid pTceFIRO into which the about 500-bp DNA fragment was inserted.

(3) Cloning of upstream and downstream portions of RNase HII gene

[0221] The nucleotide sequence of the plasmid pTceF1R0 obtained in Example 9-(2) was determined. A specific oligonucleotide TceRN-1 (SEQ ID NO:50) for cloning the upstream portion and a specific oligonucleotide TceRN-2 (SEQ ID NO:51) for cloning the downstream portion were synthesized on the basis of the determined nucleotide sequence.

[0222] PCRs were carried out in reaction mixtures containing 1 μl of the Thermococcus celer genomic DNA solution prepared in Example 9-(1) as a template, a combination of 20 pmol of TliRN-1 or 20 pmol of TliRN-2 and 20 pmol of one of the 48 primers (listed in Table 1, Example 8), 20 mM tris-acetate (pH 8.5), 50 mM potassium acetate, 3 mM magnesium acetate, 0.01% BSA, 30 μM each of dNTPs and 2.5 units of TaKaRa Ex Taq DNA polymerase (Takara Shuzo). PCRs were carried out as follows: incubation at 94°C for 3 minutes; and 40 cycles of 98°C for 10 seconds, 50°C for 10 seconds and 72°C for 40 seconds. Microcon-100 (Takara Shuzo) was used to remove primers from reaction mixtures that resulted in single bands for the PCR products and to concentrate the reaction mixtures. The concentrates were subjected to direct sequencing to screen for fragments containing the upstream or downstream portion of the RNase HII. As a result, it was shown that an about 450-bp PCR-amplified fragment TceN24 contained the upstream portion of the RNase HII gene and an about 400-bp PCR-amplified fragment TceC29 contained the downstream portion of the RNase HII gene, respectively.

(4) Cloning of entire RNase HII gene

[0223] The nucleotide sequence of a gene containing Tce RNase HII is shown in SEQ ID NO:52. The amino acid sequence of RNase HII deduced from the nucleotide sequence is shown in SEQ ID NO:53. Primers TceNde (SEQ ID NO:54) and TceBam (SEQ ID NO:55) were synthesized on the basis of the nucleotide sequence.

[0224] A PCR was carried out in a volume of 100 μl using 1 μl of the Thermococcus celer genomic DNA solution as prepared in Example 9-(1) as a template, and 20 pmol each of TceNde and TceBam as primers. Pyrobest DNA polymerase (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. An amplified DNA fragment of about 0.7 kb was digested with NdeI and BamHI (both from Takara Shuzo). Then, plasmids

pTCE265Nd and pTCE207 were constructed by incorporating the resulting DNA fragment between NdeI and BamHI sites in a plasmid vector pTV119Nd (a plasmid in which the NcoI site in pTV119N is converted into a NdeI site) or pET3a (Novagen), respectively.

(5) Determination of nucleotide sequence of DNA fragment containing RNase HII gene

[0225] The nucleotide sequences of the DNA fragments inserted into pTCE265Nd and pTCE207 obtained in Example 9-(4) were determined according to a dideoxy method.

[0226] Analyses of the determined nucleotide sequences revealed the existence of open reading frames each presumably encoding RNase HII. The nucleotide sequence of the open reading frame in pTCE207 is shown in SEQ ID NO:56. The amino acid sequence of RNase HII deduced from the nucleotide sequence is shown in SEQ ID NO:57. "A" at position 14 in the nucleotide sequence of the open reading frame in pTCE207 was replaced by "G" in the nucleotide sequence of the open reading frame in pTCE265Nd and nucleotides at positions 693 to 696 were deleted. As a result, glutamic acid at position 5 was replaced by glycine and phenylalanine at position 231 was missing in the amino acid sequence.

[0227] Escherichia coli HMS174(DE3) transformed with the plasmid pTCE207 is designated and indicated as Escherichia coli HMS174(DE3)/pTCE207, and deposited on February 22, 2001 (date of original deposit) at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566, Japan under accession number FERM BP-7694.

(6) Expression of Thermococcus celer RNase HII gene

[0228] Escherichia coli JM109 transformed with pTCE265Nd was inoculated into 10 ml of LB medium containing 100 μg/ml of ampicillin and 1 mM IPTG and cultured with shaking at 37°C overnight. After cultivation, cells collected by centrifugation were suspended in 203 μl of Buffer A and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12,000 rpm for 10 minutes was heated at 70°C for 10 minutes and then centrifuged again at 12,000 rpm for 10 minutes to collect a supernatant as a heated supernatant. Similarly, Escherichia coli HMS174(DE3) transformed with pTCE207 was inoculated into 10 ml of LB medium containing 100 μg/ml of ampicillin and cultured with shaking at 37°C overnight. After cultivation, cells collected by centrifugation were processed according to the procedure as described above to obtain a heated supernatant.

[0229] The enzymatic activities were measured for the heated supernatants as described in Example 3-(5). As a result, RNase H activities were observed for both transformants. Thus, the activity of the polypeptide of the present invention was confirmed in spite of substitution and deletion in the nucleotide sequence or the amino acid sequence.

(7) Preparation of purified RNase HII preparation

[0230] Escherichia coli JM109 transformed with pTCE265Nd obtained in Example 9-(4) was inoculated into 2 L of LB medium containing 100 μg/ml of ampicillin and cultured with shaking at 37°C for 16 hours. After cultivation, cells collected by centrifugation were suspended in 39 ml of a sonication buffer [50 mM tris-HCl (pH 8.0), 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride] and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12,000 rpm for 10 minutes was heated at 70°C for 15 minutes. It was then centrifuged again at 12,000 rpm for 20 minutes to collect a supernatant. Thus, 37.5 ml of a heated supernatant was obtained.

[0231] The heated supernatant was subjected to RESOURSE Q column (Amersham Pharmacia Biotech) equilibrated with Buffer A [50 mM tris-HCl (pH 8.0), 1 mM EDTA] and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HII flowed through the RESOURSE Q column.

[0232] The sample was subjected to RESOURSE S column (Amersham Pharmacia Biotech) equilibrated with Buffer A and eluted with a linear gradient of 0 to 500 mM NaCl using FPLC system (Amersham Pharmacia Biotech). As a result, a fraction containing RNase HII eluted with about 220 mM NaCl was obtained.

[0233] A mixture containing 3 ml of the RNase HII fraction and Buffer A added to make the NaCl concentration to 50 mM was subjected to HiTrap-heparin column (Amersham Pharmacia Biotech) equilibrated with Buffer A containing 50 mM NaCl and eluted with a linear gradient of 50 to 550 mM NaCl using FPLC system. As a result, a fraction containing RNase HII eluted with about 415 mM NaCl was obtained.

[0234] 6 ml of the RNase HII fraction was concentrated by ultrafiltration using Centricon-10. About 178 μl of the concentrate was subjected to Superose 6 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 50 mM tris-HCl (pH 8.0) containing 100 mM NaCl and 0.1 mM EDTA and eluted with the same buffer. As a result, RNase HII was eluted at a position corresponding to a molecular weight of 29.5 kilodalton. This molecular weight corresponds to that of the RNase HII in the monomeric form. The thus eluted RNase HII was used as Tce RNase HII preparation.

[0235] The enzymatic activity of the thus obtained Tce RNase HII preparation was measured as described in Example

3-(5). As a result, an RNase H activity was observed for the Tce RNase HII preparation.

Example 10

[0236] Homology searches were conducted for the amino acid sequences and nucleotide sequences from Bacillus caldotenax (hereinafter referred to as BCA), Pyrococcus furiosus (PFU), Thermotoga maritima (TMA), Archaeoglobus fulgidus (AFU), Thermococcus litoralis (TLI), Thermococcus celer (TCE) and Pyrococcus horikoshii (PHO) obtained in Examples 2 to 9. Calculation was conducted using Maximum Matching in DNASIS-Mac (Takara Shuzo) or a computer algorithm FASTA (version 3.0; Pearson, W. R. et al., Pro. Natl. Acad. Sci., 85:2444-2448, 1988) as a search program.

[0237] Homologies of the PFU amino acid sequence to the PHO, AFU, TLI and TCE amino acid sequences as determined using DNASIS were 69%, 45%, 65% and 58%, respectively. Homologies of the PFU nucleotide sequence to the PHO, AFU, TLI and TCE nucleotide sequences as determined using DNASIS were 68%, 60%, 65% and 61%, respectively.

[0238] Gene database searches were conducted for PHO, AFU and TMA using the computer algorithm FASTA. For the PHO amino acid sequence, the highest homology to an amino acid sequence presumed to be of a ribonuclease was 70% and the lowest was 20%. The homology of PHO to AFU was 50% and the homology of PHO to TMA was 35%. For the AFU amino acid sequence, the highest homology was 50% and the lowest was 25%. The amino acid sequence homology of AFU to TMA was 32%. For the TMA amino acid sequence, the highest homology was 52% and the lowest was 22%.

[0239] Gene database searches were conducted for BCA, PFU, TCE and TLI using the computer algorithm BLAST. For the BCA ribonuclease HII, BCA ribonuclease HIII, PFU, TCE and TLI amino acid sequences, the highest homologies to amino acid sequences presumed to be of ribonucleases were 43%, 46%, 68%, 70% and 64%, respectively.

[0240] In addition, the amino acid sequence homology of PHO to AFU as determined using DNASIS was also 50%. The value determined using the computer algorithm FASTA was 50% as described above. Thus, it was shown that there was no significant difference between homology values obtained using DNASIS and the computer algorithm FASTA.

Example 11

Modes of action and properties of various RNase Hs

(1) Mode of action of Bca RNase HIII

[0241] A substrate was prepared as follows in order to compare the fashions of cleavage of Bca RNase HIII and E. coli RNase HI. A PCR was carried out using a heat extract of Escherichia coli 0157 as a template, a 5' FITC-labeled chimeric primer VT2-R280N3-17 (SEQ ID NO:61) in which three nucleotides from the 3' end are RNAs, and a DNA primer VT2-F110 (SEQ ID NO:62) to obtain a DNA fragment having three RNAs in one of the two strands. Microcon-100 (Millipore) was used to remove primers from the PCR product to obtain a substrate for cleavage with RNase H.

[0242] 39.2 μl of a reaction buffer (20 mM Hepes-KOH (pH 7.8), 1% dimethyl sulfoxide, 0.01% bovine serum albumin, 100 mM potassium acetate, 4 mM magnesium acetate, 0.002% propyrenediamine) containing 0.3 pmol of the substrate was prepared. 0.8 μl of E. coli RNase HI 30 U/μl (Takara Shuzo) or a 10-fold dilution of the purified preparation of Bca RNase HIII obtained in Example 3-(5) with Buffer A was added thereto. The mixtures were reacted at 55°C for 5 or 10 minutes. After reaction, 2 μl each of the reaction mixtures was subjected to electrophoresis on denaturing 10% acrylamide gel to determined the sizes of cleaved DNA fragments.

[0243] In case of E. coli RNase HI, signals appeared at positions of 18 bases and 19 bases. The signal at the position of 19 bases decreased over time. Background at a position of 20 bases due to incomplete removal of primers also decreased over time. In case of Bca RNase HIII, a signal appeared at a position of 19 bases. No decrease in primer background was observed.

[0244] Based on the above, E. coli RNase HI cleaves at two sites between RNAs. In addition, it was considered that E. coli RNase HI had an activity of cleaving on the 5' side of a 3' RNA and further cleaving on the 5' side of a 3' RNA which was not attached to a DNA. On the other hand, it was considered that Bca RNase HIII only cleaved on the 5' side of a 3' RNA and did not cleave if DNA was not attached on the 3' side of an RNA. Thus, it was considered that the cleavage site selectivity of Bca RNase HIII was higher than that of E. coli RNase HI.

(2) Modes of action of Pfu RNase HII, Pho RNase HII and Afu RNase HII

[0245] Substrates were prepared as follows in order to analyze the fashions of cleavage of Pfu (Pyrococcus furiosus) RNase HII, Pho (Pyrococcus horikoshii) RNase HII and Afu (Archaeoglobus fulgidus) RNase HII. PCRs were carried

out using a heat extract of Escherichia coli 0157 as a template, a 5' FITC-labeled chimeric primer VT2-IF20N3 (SEQ ID NO:63) in which three nucleotides from the 3' end are RNAs, a 5' FITC-labeled chimeric primer VT2-IF19N2 (SEQ ID NO:64) in which two nucleotides from the 3' end are RNAs or a 5' FITC-labeled chimeric primer VT2-IF18N1 (SEQ ID NO:65) in which one nucleotide at the 3' end is RNA, and a DNA primer VT2IR20 (SEQ ID NO:66) to obtain DNA fragments VFN3, VFN2 and VFN1 which had three, two and one RNA(s) in one of the two strands, respectively. Microcon-100 was used to remove primers from the PCR products to obtain substrates for cleavage with RNase H.

[0246] 39 μl of a reaction buffer (20 mM Hepes-KOH (pH 7.8), 1% dimethyl sulfoxide, 0.01% bovine serum albumin, 100 mM potassium acetate, 4 mM magnesium acetate, 0.002% propyrenediamine) containing 0.3 pmol of one of the substrates was prepared. 1 μl of one of the purified preparations of the respective RNase Hs at a concentration of 37.2 U/μl was added thereto. The mixtures were reacted at 55°C for 5 or 10 minutes. After reaction, 2 μl each of the reaction mixtures was subjected to electrophoresis on denaturing 10% acrylamide gel to determined the sizes of cleaved DNA fragments.

[0247] For each of Pfu RNase HII, Pho RNase HII and Afu RNase HII, a signal was observed at a position of 19 bases using VFN3 as a substrate. For each of Pfu RNase HII, Pho RNase HII and Afu RNase HII, a signal was observed at a position of 18 bases using VFN2 as a substrate. For each of Pfu RNase HII and Pho RNase HII, a signal was observed at a position of 17 bases using VFN1 as a substrate.

[0248] Based on the above, Pfu RNase HII, Pho RNase HII and Afu RNase HII cleaved on the 5' side of a 3' RNA. Pfu RNase HII and Pho RNase HII cleaved on the 5' side of an RNA even if the number of RNA was one. There has been no report on an RNase H that cleaves even if the number of RNA is one. Since the intensities of the signals upon cleavage were similar regardless of the number of RNAs, it was shown that there was no difference in cleavage efficiency depending on the number of RNAs. In addition, since decrease in an appeared signal over time or appearance of a shorter signal was not observed, it was shown that no cleavage takes place if DNA was not attached on the 3' side of an RNA.

(3) Ion requirements of Bca RNase HII and Tma RNase HII

[0249] According to the method for measuring an RNase H activity as described in Example 1, Bca RNase HII and Tma RNase HII required Mn^{2+} and did not exhibit an activity in the presence of Mg^{2+} at all. Comparison of cleavage in the presence of Mg^{2+} or Mn^{2+} was carried out using VFN3 as described in Example 11-(2) as a substrate for them.

[0250] 39.2 μl of a reaction buffer (20 mM Hepes-KOH (pH 7.8), 1% dimethyl sulfoxide, 0.01% bovine serum albumin, 100 mM potassium acetate, 4 mM magnesium acetate, 0.002% propyrenediamine) containing 0.3 pmol of the substrate and 39.2 ul of a Mn+ reaction buffer (20 mM Hepes-KOH (pH 7.8), 1% dimethyl sulfoxide, 0.01% bovine serum albumin, 100 mM potassium acetate, 10 mM manganese chloride, 0.002% propyrenediamine) containing 0.3 pmol of the substrate were prepared. 0.8 μl of E. coli RNase HI (30 U/μl; Takara Shuzo), a 10-fold dilution of the purified preparation of Bca RNase HII (obtained in Example 2-(6)) with Buffer A or a 25-fold dilution of the crude cell extract of Tma RNase HII (obtained in Example 5-(3)) with Buffer A was added thereto. The mixtures were reacted at 55°C for 5 or 10 minutes. After reaction, 2 μl each of the reaction mixtures was subjected to electrophoresis on denaturing 10% acrylamide gel to determined the sizes of cleaved DNA fragments.

[0251] When Bca RNase HII or Tma RNase HII was used in the presence of Mg^{2+} or Mn^{2+}, a signal appeared at a position of 19 bases in each case. The levels of the signals were equivalent each other.

[0252] Bca RNase HII and Tma RNase HII did not exhibit an activity in the presence of Mg^{2+} at all according to the method for measuring an RNase H activity as described in Example 1. However, they exhibited cleavage activities in the presence of Mg^{2+} which were equivalent to those observed in the presence of Mn^{2+} as described above.

[0253] Base on the above, it was shown that the enzymes may not require Mn^{2+} for cleavage depending on the form of a substrate, it may be possible to substitute Mg^{2+} for Mn^{2+}, and a reaction in the same reaction mixture for an enzyme that requires Mn^{2+} or Ng^{2+} may be possible.

(4) Examination of thermostability of RNase H

[0254] Thermostabilities were examined using Escherichia coli transformed with the following: pRHB1 and pBCA3Nd obtained in Examples 2-(5) and 3-(4) for Bacillus caldotenax RNase H; pPFU220 obtained in Example 4-(2) for Pyrococcus furiosus RNase H; pTM-RNH obtained in Example 5-(2) for Thermotoga maritima RNase H; pPHO238 obtained in Example 6-(2) for Pyrococcus horikoshii RNase H; pAFU204 obtained in Example 7-(2) for Archaeoglobus fulgidus RNase H; pTLI204 and pTLI223Nd obtained Example 8-(4) for Thermococcus litoralis RNase H; and pTCE207 and pTCE265Nd obtained in Example 9-(4) for Thermococcus celer RNase H. The E. coli strains were cultured, crude enzyme extracts prepared from the cultures were heated at 60°C for 15 minutes, and the RNase H activities were determined according to the method as described in Example 1. As a result, RNase H activities were observed for the RNase Hs from all of the strains.

Industrial Applicability

[0255] The present invention provides a polypeptide having an RNase H activity which is highly valuable for genetic engineering, a gene encoding said polypeptide and a method for producing said polypeptide by genetic engineering. Since the RNase H of the present invention is thermostable, the present invention provide a method for producing an RNase H which is industrially advantageous.

[0256] It is now possible to use the RNase H of the present invention for various uses according to the present invention.

Sequence Listing Free Text

[0257] SEQ ID NO:1: PCR primer BsuII-3 for cloning a gene encoding a polypeptide having a RNase HII activity from Bacillus caldotenax.

[0258] SEQ ID NO:2: PCR primer BsuII-6 for cloning a gene encoding a polypeptide having a RNase HII activity from Bacillus caldotenax.

[0259] SEQ ID NO:3: PCR primer RNII-S1 for cloning a gene encoding a polypeptide having a RNase HII activity from Bacillus caldotenax.

[0260] SEQ ID NO:4: PCR primer RNII-S2 for cloning a gene encoding a polypeptide having a RNase HII activity from Bacillus caldotenax.

[0261] SEQ ID NO:5: PCR primer RNII-S5 for cloning a gene encoding a polypeptide having a RNase HII activity from Bacillus caldotenax.

[0262] SEQ ID NO:6: PCR primer RNII-S6 for cloning a gene encoding a polypeptide having a RNase HII activity from Bacillus caldotenax.

[0263] SEQ ID NO:7: PCR primer RNII-Nde for cloning a gene encoding a polypeptide having a RNase HII activity from Bacillus caldotenax.

[0264] SEQ ID NO:10: PCR primer BsuIII-1 for cloning a gene encoding a polypeptide having a RNase HIII activity from Bacillus caldotenax.

[0265] SEQ ID NO-11: PCR primer BsuIII-3 for cloning a gene encoding a polypeptide having a RNase HIII activity from Bacillus caldotenax.

[0266] SEQ ID NO:12: PCR primer BsuIII-6 for cloning a gene encoding a polypeptide having a RNase HIII activity from Bacillus caldotenax.

[0267] SEQ ID NO:13: PCR primer BsuIII-8 for cloning a gene encoding a polypeptide having a RNase HIII activity from Bacillus caldotenax.

[0268] SEQ ID NO:14: PCR primer RNIII-S3 for cloning a gene encoding a polypeptide having a RNase HIII activity from Bacillus caldotenax.

[0269] SEQ ID NO:15: PCR primer BcaRNIII-3 for cloning a gene encoding a polypeptide having a RNase HIII activity from Bacillus caldotenax.

[0270] SEQ ID NO:18: PCR primer BcaRNIIINde for amplifying a gene encoding a polypeptide having a RNase HIII activity from Bacillus caldotenax.

[0271] SEQ ID NO:20: PCR primer 1650Nde for cloning a gene encoding a polypeptide having a RNase HII activity from Pyrococcus furiosus.

[0272] SEQ ID NO:21: PCR primer 1650Bam for cloning a gene encoding a polypeptide having a RNase HII activity from Pyrococcus furiosus.

[0273] SEQ ID NO:24: PCR primer 915-F1 for cloning a gene encoding a polypeptide having a RNase HII activity from Thermotoga maritima.

[0274] SEQ ID NO:25: PCR primer 915-F2 for cloning a gene encoding a polypeptide having a RNase HII activity from Thermotoga maritima.

[0275] SEQ ID NO:26: PCR primer 915-R1 for cloning a gene encoding a polypeptide having a RNase HII activity from Thermotoga maritima.

[0276] SEQ ID NO:27: PCR primer 915-R2 for cloning a gene encoding a polypeptide having a RNase HII activity from Thermotoga maritima.

[0277] SEQ ID NO:29: PCR primer PhoNde for cloning a gene encoding a polypeptide having a RNase HII activity from Pyrococcus horikoshii.

[0278] SEQ ID NO:30: PCR primer PhoBam for cloning a gene encoding a polypeptide having a RNase HII activity from Pyrococcus horikoshii.

[0279] SEQ ID NO:34: PCR primer AfuNde for cloning a gene encoding a polypeptide having a RNase HII activity from Archaeoglobus fulgidus.

[0280] SEQ ID NO:35: PCR primer AfuBam for cloning a gene encoding a polypeptide having a RNase HII activity

from Archaeoglobus fulgidus.

[0281] SEQ ID NO:38: PCR primer RN-F1 for cloning a gene encoding a polypeptide having a RNase HII activity from Thermococcus litoralis.

[0282] SEQ ID NO:39: PCR primer RN-R0 for cloning a gene encoding a polypeptide having a RNase HII activity from Thermococcus litoralis.

[0283] SEQ ID NO:40: PCR primer TliRN-1 for cloning a gene encoding a polypeptide having a RNase HII activity from Thermococcus litoralis.

[0284] SEQ ID NO:41: PCR primer TliRN-2 for cloning a gene encoding a polypeptide having a RNase HII activity from Thermococcus litoralis.

[0285] SEQ ID NO:44: PCR primer TliNde for amplifying a gene encoding a polypeptide having a RNase HII activity from Thermococcus litoralis.

[0286] SEQ ID NO:45: PCR primer TliBam for amplifying a gene encoding a polypeptide having a RNase HIII activity from Thermococcus litoralis.

[0287] SEQ ID NO:48: PCR primer RN-F1 for cloning a gene encoding a polypeptide having a RNase HII activity from Thermococcus celer.

[0288] SEQ ID NO:49: PCR primer RN-R0 for cloning a gene encoding a polypeptide having a RNase HII activity from Thermococcus celer.

[0289] SEQ ID NO:50: PCR primer TceRN-1 for cloning a gene encoding a polypeptide having a RNase HII activity from Thermococcus celer.

[0290] SEQ ID NO:51: PCR primer TceRN-2 for cloning a gene encoding a polypeptide having a RNase HII activity from Thermococcus celer.

[0291] SEQ ID NO:54: PCR primer TceNde for amplifying a gene encoding a polypeptide having a RNase HII activity from Thermococcus celer.

[0292] SEQ ID NO:55: PCR primer TceBam for amplifying a gene encoding a polypeptide having a RNase HIII activity from Thermococcus celer.

[0293] SEQ ID NO:61: Designed chimeric oligonucleotide p rimer as VT2-R280N3-17 for amplifying a portion of vero tox in 2-encoding sequence from hemorrhagic Escherichia coli 0-157."Nucleotides 18 to 20 are ribonucleotides-other nucleot ides are deoxyribonucleotides"

[0294] SEQ ID NO:62: Designed oligonucleotide primer as VT2-F110 for amplifying a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli 0-157.

[0295] SEQ ID NO:63: Designed chimeric oligonucleotide p rimer as VT2-IF20N3 for amplifying a VFN3 from hemorrhagic Escherichia coli 0-157."Nucleotides 17 to 19 are ribonucleo tides-other nucleotides are deoxyribonucleotides"

[0296] SEQ ID NO:64: Designed chimeric oligonucleotide p rimer as VT2-IF19N2 for amplifying VFN2 from hemorrhagic Es cherichia coli 0-157."Nucleotides 16 to 18 are ribonucleoti des-other nucleotides are deoxyribonucleotides"

[0297] SEQ ID NO:65: Designed chimeric oligonucleotide p rimer as VT2-IF18N1 for amplifying a VFN1 from hemorrhagic Escherichia coli 0-157."Nucleotides 15 to 17 are ribonucleo tides-other nucleotides are deoxyribonucleotides"

[0298] SEQ ID NO:66: Designed oligonucleotide primer as VT21R20 for amplifying a portion of vero toxin 2-encoding s equence from hemorrhagic Escherichia coli 0-157.

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<120> Thermostable ribonuclease H

<130> 38-56

<150> JP 2000-280785

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<151> 2001-03-07

<160> 66

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<211> 20

<212> DNA

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<210> 3

<211> 20

<212> DNA

<213> Artificial Sequence

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cgcgcttttc cggcgtcagc   20

<210> 4

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer RNII-S2 for cloning a gene encoding a polypeptide having a RNaseHII activity from Bacillus caldotenax

<400> 4
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<210> 5
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer RNII-S5 for cloning a gene encoding a polypeptide having a RNaseHII activity from Bacillus caldotenax

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<210> 6
<211> 20
<212> DNA
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<220>
<223> PCR primer RNII-S6 for cloning a gene encoding a polypeptide having a RNaseHII activity from Bacillus caldotenax

<400> 6
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<210> 7

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer RNII-Nde for cloning a gene encoding a polypeptide having a RNaseHII activity from Bacillus caldotenax

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tagaagaggg agaggcatat gaagcggtat acggtgaaa 39

<210> 8

<211> 780

<212> DNA

<213> Bacillus caldotenax

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gtgacgcgcg accggtggat gaaagaactg gatcgccgct atccacaata cgggttcgcg 660

cgccatatgg gctacggaac gccggaacat ttcgaggcga tccgccgcta cggcgttacg 720

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<210> 9

<211> 260

<212> PRT

<213> Bacillus caldotenax

<400> 9

Met Lys Arg Tyr Thr Val Lys Asp Ile Glu Ala Leu Leu Pro Lys
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Leu Gly Ala Asp Asp Pro Arg Trp Glu Met Leu Arg Gln Asp Glu
20 25 30

Arg Lys Ser Val Gln Ala Leu Leu Ala Arg Phe Glu Arg Gln Lys
35 40 45

Ala Arg Arg His Ala Ile Glu Gln Arg Trp Glu Glu Leu Met Arg
50 55 60

Tyr Glu Arg Glu Leu Tyr Ala Ala Gly Val Arg Arg Ile Ala Gly
65 70 75

Ile Asp Glu Ala Gly Arg Gly Pro Leu Ala Gly Pro Val Val Ala
80 85 90

Ala Ala Val Ile Leu Pro Lys Asp Ala Tyr Leu Pro Gly Leu Asp
95 100 105

Asp Ser Lys Arg Leu Thr Pro Glu Lys Arg Glu Ala Leu Phe Ala
110 115 120

Gln Ile Glu Ala Cys Ala Val Ala Ile Gly Ile Gly Ile Val Ser
125 130 135

Ala Ala Glu Ile Asp Glu Arg Asn Ile Tyr Glu Ala Thr Arg Gln

140 145 150

Ala Met Ala Lys Ala Val Asn Ala Leu Ser Pro Pro Pro Glu His

155 160 165

Leu Leu Val Asp Ala Met Ala Val Pro Cys Pro Leu Pro Gln Gln

170 175 180

Arg Leu Ile Lys Gly Asp Ala Asn Ser Ala Ser Ile Ala Ala Ala

185 190 195

Ser Val Ile Ala Lys Val Thr Arg Asp Arg Trp Met Lys Glu Leu

200 205 210

Asp Arg Arg Tyr Pro Gln Tyr Gly Phe Ala Arg His Met Gly Tyr

215 220 225

Gly Thr Pro Glu His Phe Glu Ala Ile Arg Arg Tyr Gly Val Thr

230 235 240

Pro Glu His Arg Arg Ser Phe Ala Pro Val Arg Glu Val Leu Lys

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Ala Ser Glu Gln Leu

260

<210> 10

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer BsuIII-1 for cloning a gene encoding a polypeptide having a RNaseHIII activity from Bacillus caldotenax

<400> 10

ggtaaggtct tgttycargg 20

<210> 11

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer BsuIII-3 for cloning a gene encoding a polypeptide having a RNaseHIII activity from Bacillus caldotenax

<400> 11

ggaaccggag attayttygg 20

<210> 12

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer BsuIII-6 for cloning a gene encoding a polypeptide having a RNaseHIII activity from Bacillus caldotenax

<400> 12

atgattgaag cagcngcnac 20

<210> 13

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer BsuIII-8 for cloning a gene encoding a polypeptide having a RNaseHIII activity from Bacillus caldotenax

<400> 13

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<210> 14

<211> 20

<212> DNA

<213> Artificial Sequence

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<223> PCR primer RNIII-S3 for cloning a gene encoding a polypeptide having a RNaseHIII activity from Bacillus caldotenax

<400> 14

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<210> 15

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer BcaRNIII-3 for cloning a gene encoding a polypeptide having a RNaseHIII activity from Bacillus caldotenax

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gatacgtgga cactttccgc   20

<210> 16

<211> 915

<212> DNA

<213> Bacillus caldotenax

<400> 16

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tccgaccggc ttccggctgg agcgttgttt gccgtcaagc gcccggatgt cgtcatcacc   120

gcctaccgct caggcaaagt gctgtttcaa gggaaagcgg cggagcaaga agcagcgaaa   180

tggatatcag ggcgagcgc ctcaaacgaa acagctgacc accagccgtc cgctttggca   240

gctcatcaac tcgggtctct ttccgccatc ggttccgatg aagtcggcac cggcgattat   300

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gccatcatgg aaaccgtgcc gcatgcggtc accgtgttgg acaatgccga atacaaccgc   480

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ccaaaaggcg ccggcgccat tgtcgatgaa gccgcggcca acatcatccg cgcgcggggg   840

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atcgccaaac gccgg   915

<210> 17

<211> 305

<212> PRT

<213> Bacillus caldotenax

<400> 17

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Tyr Gln Asp Ala Leu Ser Asp Arg Leu Pro Ala Gly Ala Leu Phe
20 25 30

Ala Val Lys Arg Pro Asp Val Val Ile Thr Ala Tyr Arg Ser Gly
35 40 45

Lys Val Leu Phe Gln Gly Lys Ala Ala Glu Gln Glu Ala Ala Lys
50 55 60

Trp Ile Ser Gly Ala Ser Ala Ser Asn Glu Thr Ala Asp His Gln
65 70 75

Pro Ser Ala Leu Ala Ala His Gln Leu Gly Ser Leu Ser Ala Ile
80 85 90

Gly Ser Asp Glu Val Gly Thr Gly Asp Tyr Phe Gly Pro Ile Val
95 100 105

Val Ala Ala Ala Tyr Val Asp Arg Pro His Ile Ala Lys Ile Ala
110 115 120

Ala Leu Gly Val Lys Asp Ser Lys Gln Leu Asn Asp Glu Ala Ile
125 130 135

Lys Arg Ile Ala Pro Ala Ile Met Glu Thr Val Pro His Ala Val
140 145 150

Thr Val Leu Asp Asn Ala Glu Tyr Asn Arg Trp Gln Arg Ser Gly
155 160 165

Met Pro Gln Thr Lys Met Lys Ala Leu Leu His Asn Arg Thr Leu
170 175 180

Val Lys Leu Val Asp Ala Ile Ala Pro Ala Glu Pro Glu Ala Ile
185 190 195

Ile Ile Asp Glu Phe Leu Lys Arg Asp Ser Tyr Phe Arg Tyr Leu

200 205 210

Ser Asp Glu Asp Arg Ile Ile Arg Glu Arg Val His Cys Leu Pro

215 220 225

Lys Ala Glu Ser Val His Val Ser Val Ala Ala Ala Ser Ile Ile

230 235 240

Ala Arg Tyr Val Phe Leu Glu Glu Met Glu Gln Leu Ser Arg Ala

245 250 255

Val Gly Leu Leu Leu Pro Lys Gly Ala Gly Ala Ile Val Asp Glu

260 265 270

Ala Ala Ala Asn Ile Ile Arg Ala Arg Gly Ala Glu Ala Leu Glu

275 280 285

Thr Cys Ala Lys Leu His Phe Ala Asn Thr Lys Lys Ala Leu Asp

290 295 300

Ile Ala Lys Arg Arg

305

<210> 18

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer BcaRNIIINde for amplifying a gene encoding a polypeptide having a RNaseHIII activity from Bacillus caldotenax

<400> 18

cgaacgttgt caaaccatat gattcaagcc gaccaacag 39

<210> 19

<211> 663

<212> DNA

<213> Pyrococcus horikoshii

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gaagaatatt acaaacaata tggtgacttt cctccaatag ttaggagaac ttgggaaacc 600
gctaggaaga tagaggaaag gtttagaaaa aatcagctaa cgcttgataa attccttaag 660
tga 663

<210> 20

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer 1650Nde for cloning a gene encoding a polypeptide having a RNaseHII activity from Pyrococcus furiosus

<400> 20

caggaggaga gacatatgaa aatagggggga att 33

<210> 21

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer 1650Bam for cloning a gene encoding a polypeptide having a RNaseHII activity from Pyrococcus furiosus

<400> 21

gaaggttgtg gatccacttt ctaaggtttc tta 33

<210> 22

<211> 672

<212> DNA

<213> Pyrococcus furiosus

<400> 22

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tccaaacaac taacacccca tgaaaggaag aatttatttt cccagataac ctcaatagcg 180

gatgattaca aaatagtgat agtatcccca gaagaaatcg acaatagatc aggaacaatg 240

aacgagttag aggtagagaa gtttgctctc gccttaaatt cgcttcagat aaaaccagct 300

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agactcaatt ataaggcgaa gattattgcc gaacacaagg ccgatgcaaa gtatccagta 420

gtttcagcag cttcaatact tgcaaaggtt gttagggatg aggaaattga aaaattaaaa 480

aagcaatatg gagactttgg ctctgggtat ccaagtgatc caaaaaccaa gaaatggctt 540

gaagagtact acaaaaaaca caactctttc cctccaatag tcagacgaac ctgggaaact 600

gtaagaaaaa tagaggaaag cattaaagcc aaaaaatccc agctaacgct tgataaattc 660

tttaagaaac ct 672

<210> 23

<211> 224

<212> PRT

<213> Pyrococcus furiosus

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20 25 30

Glu Lys Leu Arg Asn Ile Gly Val Lys Asp Ser Lys Gln Leu Thr
35 40 45

Pro His Glu Arg Lys Asn Leu Phe Ser Gln Ile Thr Ser Ile Ala
50 55 60

Asp Asp Tyr Lys Ile Val Ile Val Ser Pro Glu Glu Ile Asp Asn
65 70 75

Arg Ser Gly Thr Met Asn Glu Leu Glu Val Glu Lys Phe Ala Leu
80 85 90

Ala Leu Asn Ser Leu Gln Ile Lys Pro Ala Leu Ile Tyr Ala Asp
95 100 105

Ala Ala Asp Val Asp Ala Asn Arg Phe Ala Ser Leu Ile Glu Arg
110 115 120

Arg Leu Asn Tyr Lys Ala Lys Ile Ile Ala Glu His Lys Ala Asp
125 130 135

Ala Lys Tyr Pro Val Val Ser Ala Ala Ser Ile Leu Ala Lys Val

140 145 150

Val Arg Asp Glu Glu Ile Glu Lys Leu Lys Lys Gln Tyr Gly Asp

155 160 165

Phe Gly Ser Gly Tyr Pro Ser Asp Pro Lys Thr Lys Lys Trp Leu

170 175 180

Glu Glu Tyr Tyr Lys Lys His Asn Ser Phe Pro Pro Ile Val Arg

185 190 195

Arg Thr Trp Glu Thr Val Arg Lys Ile Glu Glu Ser Ile Lys Ala

200 205 210

Lys Lys Ser Gln Leu Thr Leu Asp Lys Phe Phe Lys Lys Pro

215 220

<210> 24

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer 915-F1 for cloning a gene encoding a polypeptide having a RNaseHII activity from Thermotoga maritima

<400> 24

aaaaagcttg ggaatagatg agctttac 28

<210> 25

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer 915-F2 for cloning a gene encoding a polypeptide having a RNaseHII activity from Thermotoga maritima

<400> 25

aaaccatggg aatagatgag ctttac 26

<210> 26

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer 915-R1 for cloning a gene encoding a polypeptide having a RNaseHII activity from Thermotoga maritima

<400> 26

aaatctagat cctcaacttt gtcgatgtg 29

<210> 27

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer 915-R2 for cloning a gene encoding a polypeptide having a RNaseHII activity from Thermotoga maritima

<400> 27

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<210> 28

<211> 663

<212> DNA

<213> Pyrococcus horikoshii

<400> 28

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gaagaatatt acaaacaata tggtgacttt cctccaatag ttaggagaac ttgggaaacc 600
gctaggaaga tagaggaaag gtttagaaaa aatcagctaa cgcttgataa attccttaag 660
tga 663

<210> 29

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer PhoNde for cloning a gene encoding a polypeptide having a RNaseHII activity from Pyrococcus horikoshii

<400> 29

aggaggaaaa tcatatgaag gttgctggag 30

<210> 30

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer PhoBam for cloning a gene encoding a polypeptide having a RNaseHII activity from Pyrococcus horikoshii

<400> 30

ttacatgaag gatccaagat cacttaagga 30

<210> 31

<211> 673

<212> DNA

<213> Pyrococcus horikoshii

<400> 31

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tccaaacaat taactcctgg gcaacgtgaa aaactattta gcaaattaat agatatccta 180
gacgattatt atgttcttct cgttaccccc aaggaaatag atgagaggca tcattctatg 240
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gggttgaaat atgaagccac ggttatcgcc gagcataaag ccgatgcaaa gtatgagata 420

gtatcggcag catcaataat tgcaaaggtc actagggata gagagataga gaagctaaag 480

caaaagtatg gggaatttgg ttctggctat ccgagtgatc cgagaactaa ggagtggctt 540

gaagaatatt acaaacaata tggtgacttt cctccaatag ttaggagaac ttgggaaacc 600

gctaggaaga tagaggaaag gtttagaaaa aatcagctaa cgcttgataa attccttaag 660

tgatcttgga tcc 673

<210> 32

<211> 220

<212> PRT

<213> Pyrococcus horikoshii

<400> 32

Met Lys Val Ala Gly Val Asp Glu Ala Gly Arg Gly Pro Val Ile
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Gly Pro Leu Val Ile Gly Val Ala Val Ile Asp Glu Lys Asn Ile
20 25 30

Glu Arg Leu Arg Asp Ile Gly Val Lys Asp Ser Lys Gln Leu Thr
35 40 45

Pro Gly Gln Arg Glu Lys Leu Phe Ser Lys Leu Ile Asp Ile Leu
50 55 60

Asp Asp Tyr Tyr Val Leu Leu Val Thr Pro Lys Glu Ile Asp Glu
65 70 75

Arg His His Ser Met Asn Glu Leu Glu Ala Glu Lys Phe Val Val
80 85 90

Ala Leu Asn Ser Leu Arg Ile Lys Pro Gln Lys Ile Tyr Val Asp
95 100 105

Ser Ala Asp Val Asp Pro Lys Arg Phe Ala Ser Leu Ile Lys Ala
110 115 120

Gly Leu Lys Tyr Glu Ala Thr Val Ile Ala Glu His Lys Ala Asp

125 130 135

Ala Lys Tyr Glu Ile Val Ser Ala Ala Ser Ile Ile Ala Lys Val

140 145 150

Thr Arg Asp Arg Glu Ile Glu Lys Leu Lys Gln Lys Tyr Gly Glu

155 160 165

Phe Gly Ser Gly Tyr Pro Ser Asp Pro Arg Thr Lys Glu Trp Leu

170 175 180

Glu Glu Tyr Tyr Lys Gln Tyr Gly Asp Phe Pro Pro Ile Val Arg

185 190 195

Arg Thr Trp Glu Thr Ala Arg Lys Ile Glu Glu Arg Phe Arg Lys

200 205 210

Asn Gln Leu Thr Leu Asp Lys Phe Leu Lys

215 220

<210> 33

<211> 626

<212> DNA

<213> Archaeoglobus fulgidus

<400> 33

atgaaggcag gcatcgatga ggctggaaag ggctgcgtca tcggcccact ggttgttgca 60

ggagtggctt gcagcgatga ggataggctg agaaagcttg gtgtgaaaga ctccaaaaag 120

ctaagtcagg ggaggagaga ggaactagcc gaggaaataa ggaaaatctg cagaacggag 180

gttttgaaag tttctcccga aaatctcgac gaaaggatgg ctgctaaaac cataaacgag 240

attttgaagg agtgctacgc tgaaataatt ctcaggctga agccggaaat tgcttatgtt 300

gacagtcctg atgtgattcc cgagagactt tcgagggagc ttgaggagat tacggggttg 360

agagttgtgg ccgagcacaa ggcggacgag aagtatcccc tggtagctgc ggcttcaatc 420

atcgcaaagg tggaaaggga gcgggagatt gagaggctga aagaaaaatt cggggatttc 480

ggcagcggct atgcgagcga tccgaggaca agagaagtgc tgaaggagtg gatagcttca 540

ggcagaattc cgagctgcgt gagaatgcgc tggaagacgg tgtcaaatct gaggcagaag 600

acgcttgacg atttctaaac gaaacc 626

<210> 34

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer AfuNde for cloning a gene encoding a polypeptide having a RNaseHII activity from Archaeoglobus fulgidus

<400> 34

aagctgggtt tcatatgaag gcaggcatcg 30

<210> 35

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer AfuBam for cloning a gene encoding a polypeptide having a RNaseHII activity from Archaeoglobus fulgidus

<400> 35

tggtaataac ggatccgttt agaaatcgtc 30

<210> 36

<211> 638

<212> DNA

<213> Archaeoglobus fulgidus

<400> 36

catatgaagg caggcatcga tgaggctgga aagggctgcg tcatcggccc actggttgtt 60

gcaggagtgg cttgcagcga tgaggatagg ctgagaaagc ttggtgtgaa agactccaaa 120

aagctaagtc aggggaggag agaggaacta gccgaggaaa taaggaaaat ctgcagaacg 180

gaggttttga aagtttctcc cgaaaatctc gacgaaagga tggctgctaa aaccataaac 240

gagattttga aggagtgcta cgctgaaata attctcaggc tgaagccgga aattgcttat 300

gttgacagtc ctgatgtgat tcccgagaga ctttcgaggg agcttgagga gattacgggg 360

ttgagagttg tggccgagca caaggcggac gagaagtatc ccctggtagc tgcggcttca 420

atcatcgcaa aggtggaaag ggagcgggag attgagaggc tgaaagaaaa attcggggat 480

ttcggcagcg gctatgcgag cgatccgagg acaagagaag tgctgaagga gtggatagct 540

tcaggcagaa ttccgagctg cgtgagaatg cgctggaaga cggtgtcaaa tctgaggcag 600

aagacgcttg acgatttcta aacggatccc cgggtacc 638

<210> 37

<211> 205

<212> PRT

<213> Archaeoglobus fulgidus

<400> 37

Met Lys Ala Gly Ile Asp Glu Ala Gly Lys Gly Cys Val Ile Gly
1 5 10 15

Pro Leu Val Val Ala Gly Val Ala Cys Ser Asp Glu Asp Arg Leu
20 25 30

Arg Lys Leu Gly Val Lys Asp Ser Lys Lys Leu Ser Gln Gly Arg
35 40 45

Arg Glu Glu Leu Ala Glu Glu Ile Arg Lys Ile Cys Arg Thr Glu

50 55 60

Val Leu Lys Val Ser Pro Glu Asn Leu Asp Glu Arg Met Ala Ala

65 70 75

Lys Thr Ile Asn Glu Ile Leu Lys Glu Cys Tyr Ala Glu Ile Ile

80 85 90

Leu Arg Leu Lys Pro Glu Ile Ala Tyr Val Asp Ser Pro Asp Val

95 100 105

Ile Pro Glu Arg Leu Ser Arg Glu Leu Glu Glu Ile Thr Gly Leu

110 115 120

Arg Val Val Ala Glu HisLys Ala Asp Glu Lys Tyr Pro Leu Val

125 130 135

Ala Ala Ala Ser Ile Ile Ala Lys Val Glu Arg Glu Arg Glu Ile

140 145 150

Glu Arg Leu Lys Glu Lys Phe Gly Asp Phe Gly Ser Gly Tyr Ala

155 160 165

Ser Asp Pro Arg Thr Arg Glu Val Leu Lys Glu Trp Ile Ala Ser

170 175 180

Gly Arg Ile Pro Ser Cys Val Arg Met Arg Trp Lys Thr Val Ser

185 190 195

Asn Leu Arg Gln Lys Thr Leu Asp Asp Phe

200 205

<210> 38

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer RN-F1 for cloning a gene encoding a polypeptide having a RNaseHII

activity from Thermococcus litoralis

<400> 38

ggcattgatg aggctggnar rgg 23

<210> 39

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer RN-R0 for cloning a gene encoding a polypeptide having a RNaseHII activity from Thermococcus litoralis

<400> 39

gtccttggat cgctgggrta ncc 23

<210> 40

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer TliRN-1 for cloning a gene encoding a polypeptide having a RNaseHII activity from Thermococcus litoralis

<400> 40

tagctttttt gaatctttga ctcc 24

<210> 41

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer TliRN-2 for cloning a gene encoding a polypeptide having a RNaseHII activity from Thermococcus litoralis

<400> 41

ctgctgcatc aatactagct aaag 24

<210> 42

<211> 675

<212> DNA

<213> Thermococcus litoralis

<400> 42

atgaagctgg gaggaataga tgaagccggc aggggaccag ttataggccc tcttgtaatt 60

gcagcggttg ttgtcgatga atcccgtatg caggagcttg aagctttggg agtcaaagat 120

tcaaaaaagc taacaccaaa aagaagagaa gagctatttg aggagattgt gcaaatagtt 180

gatgaccacg ttatcattca gctttcccca gaggagatag acggcagaga tggtacaatg 240

aacgagcttg aaattgaaaa ctttgccaaa gcgttgaact cccttaaagt taagccggat 300

gtgctctaca tagatgcggc cgatgtcaag gaaaagcgct ttggcgacat tataggtgaa 360

agactttcct tctctccaaa gataatcgcc gaacataagg cagattcaaa gtacattcca 420

gtggctgctg catcaatact agctaaagtt acccgtgaca gggcaataga gaagctcaag 480

gagctttatg gggagatagg ctcaggatat ccaagtgatc caaatacaag gaggtttctg 540

gaggagtatt acaaggctca tggggaattc cccccaatag tgaggaaaag ctggaagacc 600

cttagaaaga tagaagaaaa actaaaagct aaaaagactc agcccactat cttggacttc 660

ttaaaaaagc cttaa 675

<210> 43

<211> 224

<212> PRT

<213> Thermococcus litoralis

<400> 43

Met Lys Leu Gly Gly Ile Asp Glu Ala Gly Arg Gly Pro Val Ile
1 5 10 15

Gly Pro Leu Val Ile Ala Ala Val Val Val Asp Glu Ser Arg Met
20 25 30

Gln Glu Leu Glu Ala Leu Gly Val Lys Asp Ser Lys Lys Leu Thr
35 40 45

Pro Lys Arg Arg Glu Glu Leu Phe Glu Glu Ile Val Gln Ile Val
50 55 60

Asp Asp His Val Ile Ile Gln Leu Ser Pro Glu Glu Ile Asp Gly
65 70 75

Arg Asp Gly Thr Met Asn Glu Leu Glu Ile Glu Asn Phe Ala Lys
80 85 90

Ala Leu Asn Ser Leu Lys Val Lys Pro Asp Val Leu Tyr Ile Asp
95 100 105

Ala Ala Asp Val Lys Glu Lys Arg Phe Gly Asp Ile Ile Gly Glu
110 115 120

Arg Leu Ser Phe Ser Pro Lys Ile Ile Ala Glu His Lys Ala Asp
125 130 135

Ser Lys Tyr Ile Pro Val Ala Ala Ala Ser Ile Leu Ala Lys Val
140 145 150

Thr Arg Asp Arg Ala Ile Glu Lys Leu Lys Glu Leu Tyr Gly Glu

155 160 165

Ile Gly Ser Gly Tyr Pro Ser Asp Pro Asn Thr Arg Arg Phe Leu

170 175 180

Glu Glu Tyr Tyr Lys Ala His Gly Glu Phe Pro Pro Ile Val Arg

185 190 195

Lys Ser Trp Lys Thr Leu Arg Lys Ile Glu Glu Lys Leu Lys Ala

200 205 210

Lys Lys Thr Gln Pro Thr Ile Leu Asp Phe Leu Lys Lys Pro

215 220

<210> 44

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer TliNde for amplifying a gene encoding a polypeptide having a RNaseHII activity from Thermococcus litoralis

<400> 44

gaggaggtag gcatatgaag ctgggaggaa tagatgaag 39

<210> 45

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer TliBam for amplifying a gene encoding a polypeptide having a

RNaseHIII activity from Thermococcus litoralis

<400> 45

aaaggaaacc ttcggatcca ttaaggcttt tttaagaag 39

<210> 46

<211> 675

<212> DNA

<213> Thermococcus litoralis

<400> 46

atgaagctgg gaggaataga tgaagccggc aggggaccag ttataggccc tcttgtaatt 60
gcagcggttg ttgtcgatga atcccgtatg caggagcttg aagctttggg agtcaaagat 120
tcaaaaaagc taacaccaaa aagaagagaa gagctatttg aggagattgt gcaaatagtt 180
gatgaccacg ttatcattca gctttcccca gaggagatag acggcagaga tggtacaatg 240
aacgagcttg aaattgaaaa ctttgccaaa gcgttgaact cccttaaagt taagccggat 300
gtgctctaca tagatgcggc cgatgtcaag gaaaagcgct ttggcgacat tataggtgaa 360
agactttcct tctctccaaa gataatcgcc gaacataagg cagattcaaa gtacattcca 420
gtggctgctg catcaatact agctaaagtt acccgtgaca gggcaataga gaagctcaag 480
gagttttatg gggagatagg ctcaggatat ccaagtgatc caattacaag gaggtttctg 540
gaggagtatt acaaggctca tggggaattc cccccaatag tgaggaaaag ctggaagacc 600
cttagaaaga tagaagaaaa actaaaagct aaaaagactc agcccactat cttggacttc 660
ttaaaaaagc cttaa 675

<210> 47

<211> 224

<212> PRT

<213> Thermococcus litoralis

<400> 47

Met Lys Leu Gly Gly Ile Asp Glu Ala Gly Arg Gly Pro Val Ile

1 5 10 15

Gly Pro Leu Val Ile Ala Ala Val Val Val Asp Glu Ser Arg Met

20 25 30

Gln Glu Leu Glu Ala Leu Gly Val Lys Asp Ser Lys Lys Leu Thr

35 40 45

Pro Lys Arg Arg Glu Glu Leu Phe Glu Glu Ile Val Gln Ile Val

50 55 60

Asp Asp His Val Ile Ile Gln Leu Ser Pro Glu Glu Ile Asp Gly

65 70 75

Arg Asp Gly Thr Met Asn Glu Leu Glu Ile Glu Asn Phe Ala Lys

80 85 90

Ala Leu Asn Ser Leu Lys Val Lys Pro Asp Val Leu Tyr Ile Asp

95 100 105

Ala Ala Asp Val Lys Glu Lys Arg Phe Gly Asp Ile Ile Gly Glu

110 115 120

Arg Leu Ser Phe Ser Pro Lys Ile Ile Ala Glu His Lys Ala Asp

125 130 135

Ser Lys Tyr Ile Pro Val Ala Ala Ala Ser Ile Leu Ala Lys Val

140 145 150

Thr Arg Asp Arg Ala Ile Glu Lys Leu Lys Glu Phe Tyr Gly Glu

155 160 165

Ile Gly Ser Gly Tyr Pro Ser Asp Pro Ile Thr Arg Arg Phe Leu

170 175 180

Glu Glu Tyr Tyr Lys Ala His Gly Glu Phe Pro Pro Ile Val Arg

185 190 195

Lys Ser Trp Lys Thr Leu Arg Lys Ile Glu Glu Lys Leu Lys Ala

200 205 210

Lys Lys Thr Gln Pro Thr Ile Leu Asp Phe Leu Lys Lys Pro

215 220

<210> 48

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer RN-F1 for cloning a gene encoding a polypeptide having a RNaseHII activity from Thermococcus celer

<400> 48

ggcattgatg aggctggnar rgg 23

<210> 49

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer RN-R0 for cloning a gene encoding a polypeptide having a RNaseHII activity from Thermococcus celer

<400> 49

gtccttggat cgctgggrta ncc 23

<210> 50

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer TceRN-1 for cloning a gene encoding a polypeptide having a RNaseHII activity from Thermococcus celer

<400> 50

tctctgagct tcggaacgtt cttc   24

<210> 51

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer TceRN-2 for cloning a gene encoding a polypeptide having a RNaseHII activity from Thermococcus celer

<400> 51

acccgtgaca gggcgataga aaag   24

<210> 52

<211> 702

<212> DNA

<213> Thermococcus celer

<400> 52

ttgaagctcg caggaataga cgaggctgga aggggccccg taatcggccc gatggtcatc   60

gcggccgtcg tcctcgatga gaagaacgtt ccgaagctca gagatctcgg cgtcagggac 120

tcgaaaaagc tgaccccaaa gaggagggag agattattta acgacataat taaacttttg 180

gatgattatg taattcttga attatggccg gaggagatag actcccgcgg cgggacgctt 240

aacgagctcg aggtggagag gttcgtggag gccctcaact cgcttaaggt gaagcccgac 300

gtcgtttaca tagacgcggc ggacgtgaag gagggccgct ttggcgagga gataaaggaa 360

aggttgaact tcgaggcgaa gattgtctca gagcacaggg cggacgataa gtttttaccg 420

gtgtcctctg cctcgatact ggcgaaggtg acccgtgaca gggcgataga aaagctcaag 480

gagaagtacg gcgagatcgg gagcggctac ccgagcgacc caaggacgag ggagttcctc 540

gagaactact acagacaaca cggcgagttc ccgcccgtag tccggcgaag ctggaagacg 600

ctgagaaaga tagaggaaaa gctgaggaaa gaggccgggt caaaaaaccc ggagaattca 660

aaggaaaagg gacagacgag cctggacgta tttttgaggt ag 702

<210> 53

<211> 233

<212> PRT

<213> Thermococcus celer

<400> 53

Leu Lys Leu Ala Gly Ile Asp Glu Ala Gly Arg Gly Pro Val Ile
1 5 10 15

Gly Pro Met Val Ile Ala Ala Val Val Leu Asp Glu Lys Asn Val
20 25 30

Pro Lys Leu Arg Asp Leu Gly Val Arg Asp Ser Lys Lys Leu Thr
35 40 45

Pro Lys Arg Arg Glu Arg Leu Phe Asn Asp Ile Ile Lys Leu Leu
50 55 60

Asp Asp Tyr Val Ile Leu Glu Leu Trp Pro Glu Glu Ile Asp Ser
65 70 75

Arg Gly Gly Thr Leu Asn Glu Leu Glu Val Glu Arg Phe Val Glu

80 85 90

Ala Leu Asn Ser Leu Lys Val Lys Pro Asp Val Val Tyr Ile Asp

95 100 105

Ala Ala Asp Val Lys Glu Gly Arg Phe Gly Glu Glu Ile Lys Glu

110 115 120

Arg Leu Asn Phe Glu Ala Lys Ile Val Ser Glu His Arg Ala Asp

125 130 135

Asp Lys Phe Leu Pro Val Ser Ser Ala Ser Ile Leu Ala Lys Val

140 145 150

Thr Arg Asp Arg Ala Ile Glu Lys Leu Lys Glu Lys Tyr Gly Glu

155 160 165

Ile Gly Ser Gly Tyr Pro Ser Asp Pro Arg Thr Arg Glu Phe Leu

170 175 180

Glu Asn Tyr Tyr Arg Gln His Gly Glu Phe Pro Pro Val Val Arg

185 190 195

Arg Ser Trp Lys Thr Leu Arg Lys Ile Glu Glu Lys Leu Arg Lys

200 205 210

Glu Ala Gly Ser Lys Asn Pro Glu Asn Ser Lys Glu Lys Gly Gln

215 220 225

Thr Ser Leu Asp Val Phe Leu Arg

230

<210> 54

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer TceNde for amplifying a gene encoding a polypeptide having a

RNaseHII activity from Thermococcus celer

<400> 54

caggggggtga gcatatgaag ctcgcaggaa tagacgagg 39

<210> 55

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer TceBam for amplifying a gene encoding a polypeptide having a RNaseHII activity from Thermococcus celer

<400> 55

tgaacccgcg taggatccta cctcaaaaat acgtccagg 39

<210> 56

<211> 702

<212> DNA

<213> Thermococcus celer

<400> 56

atgaagctcg cagaaataga cgaggctgga aggggccccg taatcggccc gatggtcatc 60

gcggccgtcg tcctcgatga gaagaacgtt ccgaagctca gagatctcgg cgtcagggac 120

tcgaaaaagc tgaccccaaa gaggagggag agattattta acgacataat taaacttttg 180

gatgattatg taattcttga attatggccg gaggagatag actcccgcgg cgggacgctt 240

aacgagctcg aggtggagag gttcgtggag gccctcaact cgcttaaggt gaagcccgac 300

gtcgtttaca tagacgcggc ggacgtgaag gagggccgct ttggcgagga gataaaggaa 360

aggttgaact tcgaggcgaa gattgtctca gagcacaggg cggacgataa gtttttaccg 420

gtgtcctctg cctcgatact ggcgaaggtg acccgtgaca gggcgataga aaagctcaag 480

gagaagtacg gcgagatcgg gagcggctac ccgagcgacc caaggacgag ggagttcctc 540

gagaactact acagacaaca cggcgagttc ccgcccgtag tccggcgaag ctggaagacg 600

ctgagaaaga tagaggaaaa gctgaggaaa gaggccgggt caaaaaaccc ggagaattca 660

aaggaaaagg gacagacgag cctggacgta tttttgaggt ag 702

<210> 57

<211> 233

<212> PRT

<213> Thermococcus celer

<400> 57

Met Lys Leu Ala Glu Ile Asp Glu Ala Gly Arg Gly Pro Val Ile
1 5 10 15

Gly Pro Met Val Ile Ala Ala Val Val Leu Asp Glu Lys Asn Val
20 25 30

Pro Lys Leu Arg Asp Leu Gly Val Arg Asp Ser Lys Lys Leu Thr
35 40 45

Pro Lys Arg Arg Glu Arg Leu Phe Asn Asp Ile Ile Lys Leu Leu
50 55 60

Asp Asp Tyr Val Ile Leu Glu Leu Trp Pro Glu Glu Ile Asp Ser
65 70 75

Arg Gly Gly Thr Leu Asn Glu Leu Glu Val Glu Arg Phe Val Glu
80 85 90

Ala Leu Asn Ser Leu Lys Val Lys Pro Asp Val Val Tyr Ile Asp
95 100 105

Ala Ala Asp Val Lys Glu Gly Arg Phe Gly Glu Glu Ile Lys Glu
110 115 120

Arg Leu Asn Phe Glu Ala Lys Ile Val Ser Glu His Arg Ala Asp

125 130 135

Asp Lys Phe Leu Pro Val Ser Ser Ala Ser Ile Leu Ala Lys Val

140 145 150

Thr Arg Asp Arg Ala Ile Glu Lys Leu Lys Glu Lys Tyr Gly Glu

155 160 165

Ile Gly Ser Gly Tyr Pro Ser Asp Pro Arg Thr Arg Glu Phe Leu

170 175 180

Glu Asn Tyr Tyr Arg Gln His Gly Glu Phe Pro Pro Val Val Arg

185 190 195

Arg Ser Trp Lys Thr Leu Arg Lys Ile Glu Glu Lys Leu Arg Lys

200 205 210

Glu Ala Gly Ser Lys Asn Pro Glu Asn Ser Lys Glu Lys Gly Gln

215 220 225

Thr Ser Leu Asp Val Phe Leu Arg

230

<210> 58

<211> 714

<212> DNA

<213> Thermotoga maritima

<400> 58

atgggaatag atgagcttta caaaaaagag tttggaatcg tagcaggtgt ggatgaagcg 60

ggaagagggt gcctcgcagg tcccgttgtg gcggccgctg tcgttctgga aaaagaaata 120

gaaggaataa acgattcaaa acagctttcc cctgcgaaga gggaaagact tttagatgaa 180

ataatggaga aggcagcagt tgggttagga attgcgtctc cagaggaaat agatctctac 240

aacatattca atgccacaaa acttgctatg aatcgagcac tggagaacct gtctgtgaaa 300

ccatcatttg tactcgttga cgggaaagga atcgagttga gcgttcccgg tacatgctta 360

gtgaagggag accagaaaag caaattgata ggagcagctt ccattgttgc gaaggtcttc 420

agagatagat tgatgagcga gtttcacagg atgtatccac agttttcctt ccacaaacac 480

aaaggttacg ccacaaaaga acatctgaac gaaatcagaa agaacggagt tttaccaatc 540

caccggctga gttttgaacc tgttttagaa cttctgaccg atgatttgtt gagggagttc 600

ttcgaaaaag gcctcatctc cgaaaatcga ttcgaacgaa tattgaatct tctgggggcg 660

agaaaaagtg tggttttccg gaaagaaaga acaaaccata atctccctct tttt 714

<210> 59

<211> 238

<212> PRT

<213> Thermotoga maritima

<400> 59

Met Gly Ile Asp Glu Leu Tyr Lys Lys Glu Phe Gly Ile Val Ala
1 5 10 15

Gly Val Asp Glu Ala Gly Arg Gly Cys Leu Ala Gly Pro Val Val
20 25 30

Ala Ala Ala Val Val Leu Glu Lys Glu Ile Glu Gly Ile Asn Asp
35 40 45

Ser Lys Gln Leu Ser Pro Ala Lys Arg Glu Arg Leu Leu Asp Glu
50 55 60

Ile Met Glu Lys Ala Ala Val Gly Leu Gly Ile Ala Ser Pro Glu
65 70 75

Glu Ile Asp Leu Tyr Asn Ile Phe Asn Ala Thr Lys Leu Ala Met
80 85 90

Asn Arg Ala Leu Glu Asn Leu Ser Val Lys Pro Ser Phe Val Leu
95 100 105

Val Asp Gly Lys Gly Ile Glu Leu Ser Val Pro Gly Thr Cys Leu
110 115 120

Val Lys Gly Asp Gln Lys Ser Lys Leu Ile Gly Ala Ala Ser Ile

125 130 135

Val Ala Lys Val Phe Arg Asp Arg Leu Met Ser Glu Phe His Arg

140 145 150

Met Tyr Pro Gln Phe Ser Phe His Lys His Lys Gly Tyr Ala Thr

155 160 165

Lys Glu His Leu Asn Glu Ile Arg Lys Asn Gly Val Leu Pro Ile

170 175 180

His Arg Leu Ser Phe Glu Pro Val Leu Glu Leu Leu Thr Asp Asp

185 190 195

Leu Leu Arg Glu Phe Phe Glu Lys Gly Leu Ile Ser Glu Asn Arg

200 205 210

Phe Glu Arg Ile Leu Asn Leu Leu Gly Ala Arg Lys Ser Val Val

215 220 225

Phe Arg Lys Glu Arg Thr Asn His Asn Leu Pro Leu Phe

230 235

<210> 60

<211> 17

<212> DNA

<213> Artificial Sequence

<220>

<223> Designed oligonucleotide as tag sequence.

<400> 60

ggcacgattc gataacg 17

<210> 61

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Designed chimeric oligonucleotide primer as VT2-R280N3-I7 for amplifying a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli 0-157. "Nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

<400> 61

tgctcaataa tcanacgaag 20

<210> 62

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Designed oligonucleotide primer as VT2-F110 for amplifying a portion of ver o toxin 2-encoding sequence from hemorrhagic Escherichia coli 0-157.

<400> 62

tcgttaaata gtatacggga ag 22

<210> 63

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> Designed chimeric oligonucleotide primer as VT2-IF20N3 for amplifying a V FN3 from hemorrhagic Escherichia coli 0-157."Nucleotides 17 to 19 are ribonucleotid es-other nucleotides are deoxyribonucleotides"

<400> 63

tactgggttt ttcttcgua 19

<210> 64

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Designed chimeric oligonucleotide primer as VT2-IF19N2 for amplifying VF N2 from hemorrhagic Escherichia coli 0-157."Nucleotides 16 to 18 are ribonucleotide s-other nucleotides are deoxyribonucleotides"

<400> 64

tactgggttt ttcttcgu 18

<210> 65

<211> 17

<212> DNA

<213> Artificial Sequence

<220>

<223> Designed chimeric oligonucleotide primer as VT2-IF18N1 for amplifying a V FN1 from hemorrhagic Escherichia coli 0-157."Nucleotides 15 to 17 are ribonucleotid

es-other nucleotides are deoxyribonucleotides"

<400> 65

tactgggttt ttcttcg 17

<210> 66

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Designed oligonucleotide primer as VT21R20 for amplifying a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli 0-157.

<400> 66

gtcccctgag atatatgttc 20

Claims

1. A polypeptide having a thermostable ribonuclease H activity, which is selected from the group consisting of:

   (a) a polypeptide having the amino acid sequence of SEQ ID NO:9, 17, 23, 32, 37, 47, 57 or 59, or a portion thereof;
   (b) a polypeptide having an amino acid sequence in which at least one amino acid residue is deleted, added, inserted or substituted in the amino acid sequence of SEQ ID NO:9, 17, 23, 32, 37, 47, 57 or 59; and
   (c) a polypeptide having an amino acid sequence that shares at least 71% homology with the amino acid sequence of SEQ ID NO:9, 17, 23, 32, 37, 47, 57 or 59.

2. A nucleic acid encoding a polypeptide having a thermostable ribonuclease H activity, which is selected from the group consisting of:

   (a) a nucleic acid encoding a polypeptide having the amino acid sequence of SEQ ID NO:9, 17, 23, 32, 37, 47, 57 or 59, or a portion thereof;
   (b) a nucleic acid encoding a polypeptide having an amino acid sequence in which at least one amino acid residue is deleted, added, inserted or substituted in the amino acid sequence of SEQ ID NO:9 17, 23, 32, 37, 47, 57 or 59;
   (c) a nucleic acid having the nucleotide sequence of SEQ ID NO:8, 16, 22, 31, 36, 46, 56 or 58;
   (d) a nucleic acid in which at least one nucleotide is deleted, added, inserted or substituted in the nucleotide sequence of SEQ ID NO:8, 16, 22, 31, 36, 46, 56 or 58 such that the deletion, addition, insertion or substitution

of the nucleotide results in translation into an amino acid sequence;
(e) a nucleic acid that is hybridizable to any one of the nucleic acids of (a) to (d) or complementary strands thereof under stringent conditions; and
(f) a nucleic acid having a nucleotide sequence that shares at least 69% homology with the nucleotide sequence of SEQ ID NO:8, 16, 22, 31, 36, 46, 56 or 58.

3. A recombinant DNA comprising the nucleic acid defined by claim 2.

4. A transformant transformed with the recombinant DNA defined by claim 3.

5. A method for producing a polypeptide having a thermostable ribonuclease H activity, the method comprising:

culturing the transformant defined by claim 4; and
collecting a polypeptide having a thermostable ribonuclease H activity from the culture.

6. A polypeptide having a thermostable ribonuclease H activity, which is obtainable by culturing a transformant into which any one of the plasmids pRHB11 (FERM BP-7655), pBCA3Nd2 (FERM BP-7653), pPFU220 (FERM BP-7654), pTM-RNH (FERM BP-7652), pPH0238 (FERM BP-7692), pAFU204 (FERM BP-7691), pTLI204 (FERM BP-7693) and pTCE207 (FERM BP-7694) is transferred.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP01/07930

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] C12N15/55, C12N9/22, C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] C12N15/55, C12N9/22, C12N1/21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
JICST FILE (JOIS), WPI (DIALOG), BIOSIS (DIALOG), MEDLINE (STN), DDBJ/Genbank/EMBL/PIR/SwissProt/Geneseq

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	H. P. KLENK et al., "The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*", Nature, (1997), Vol.390, No.6665, pages 364 to 370 & H. P. KLENK et al., "Ribonuclease HII (EC 3.1.26.4), SwissProt, 30 May, 2000 (30.05.00), Accession 029634, Gene AF0621 & H. P. KLENK et al., "Archaeoglobis fulgidus section 45 of 172 of the complete genome", Genbank, 15 December, 1997 (15.12.97), Accession AE001062, Gene AF0621	1-6
X	Y. KAWARABAYASHI et al., "Complete Sequence and Gene Organization of Genome of a Hyper-thermophilic Archaebacterium, *Pyrococcus horikoshii* OT3", DNA Res., (1998), Vol.5, No.2, pages 55 to 76 & Y. KAWARABAYASHI et al., "Ribonuclease HII (EC 3.1.26.4), SwissProt, 30 May, 2000 (30.05.00), Accession 059351, Gene PH1650 & Y. KAWARABAYASHI et al., "Pyrococcus horikoshii OT3 genomic DNA, 1166001-1485000 nt. Position (6/7)", EMBL, 06 April, 2000 (06.04.00), Accession AP000006, Gene PH1650	1-6

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 November, 2001 (29.11.01)	11 December, 2001 (11.12.01)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP01/07930

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	K. E. NELSON et al., "Evidence for lateral gene transfer between Archaea and Bacteria from genome sequence of *Thermotoga maritima*, Nature, (1999), Vol.399, No.6734, pages 323 to 329 & K. E. NELSON et al., "Ribonuclease HII (EC 3.1.26.4), SwissProt, 30 May, 2000 (30.05.00), Accession Q9X017, Gene TM0915 & K. E. NELSON et al., "Thermotoga maritima section 67 of 136 of the complete genome", Genbank, 02 June, 1999 (02.06.99), Accession AE001755, Gene TM0915	1-6
X	M. HARUKI et al., "Gene Cloning and Characterization of Recombinant Rnase HII from a Hyperthermophilic Archaeon", J. Bacteriol., (1998), Vol.180, No.23, pages 6207 to 6214 (Fig. 1)	1-6
X	JP 11-32772 A (Mitsubishi Chem. Corp.), 09 February, 1999 (09.02.99), (Sequence Nos. 1, 2) (Family: none)	1-6
X	US 5610066 A (Amersham Life Sci. Inc.), 11 March, 1997 (11.03.97), (Sequence No. 1), (Family: none)	1-6

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP01/07930

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

(See extra sheet.)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP01/07930

Continuation of Box No. II of continuation of first sheet (1)

DNAs according to SEQ ID NOS:8, 16, 22, 31, 36, 46, 56 and 58 given in Sequence Listing as set forth in claim 1 neither have any chemical structure in common nor originate in a specific organism. The only one matter common to these DNAs resides in that they all encode an enzyme having a thermotolerant ribonuclease H activity.

However, Document 1 (JP 11-32772 A, Mitsubishi Chemical Industry, 9, February, 1999 (09.02.1999)) discloses a DNA encoding an enzyme having a thermotolerant ribonuclease activity and originating in a hyperthemophilic archaebacterium *Pyrococcus sp. KOD1* (Claim, SEQ ID NOS:1 and 2).

Accordingly, being a DNA encoding an enzyme having a thermotolerant ribonuclease H activity cannot be regarded as a special technical matter as defined in Rule 13.2 of the Regulations under the PCT.

Such being the case, the inventions relating to the DNAs according to SEQ ID NOS:8, 16, 22, 31, 36, 46, 56 and 58, among the inventions as set forth in claim 1, are considered not as relating to a group of inventions so linked as to form a single general inventive concept but as being groups of inventions consisting of 8 inventions respectively relating to 8 different proteins.

Therefore, there is no special technical feature common to all of the claims. Such being the case, the inventions as set forth in claims 1 to 6 involve 8 groups of inventions relating respectively to the DNAs according to SEQ ID NOS:8, 16, 22, 31, 36, 46, 56 and 58 as set forth in claim 1.

Form PCT/ISA/210 (extra sheet) (July 1992)